STUDIES ON THE NATURE AND APPLICATIONS OF ANTIBODY-DERIVED BINDING-SITE MOLECULES.

A thesis submitted for the degree of Ph.D.,

by

Michael John McMahon B.Sc.,

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Based on research carried out at

School of Biotechnology,

Dublin City University,

Dublin 9,

Ireland.

Under the supervision of

Professor Richard O'Kennedy.

To

My parents

I hereby certify that this material, which I now submit for assessment on the programme leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Michael McNulty

Dated: 20-06-99

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CONTENTS:

PRESENTATIONS:

McMahon, M. and R O'Kennedy, Natural selection of therapeutic intrabodies from combinatorial libraries, Presented at: Discovery and development of novel therapeutic agents for the 21st century, Keystone Symposia on molecular and cellular biology, Tamarron, Colorado, March 16-21, 1997.

McMahon, M. and R. O'Kennedy, Development of diverse intrabody libraries for functional genomic analysis, Presented at: Biochemical Society Meeting, Dublin City University, 9-10 Sept., 1998.

ABBREVIATIONS AND SYMBOLS:

Ab	*Antibody*
Ag	*Antigen*
AIDS	*Acquired immune deficiency syndrome*
AMP	*2-amino-2-methyl-1-propanol*
ASC	*Antibody secreting cells*
BCIP	*5-bromo-4-chloro-3-indolyl phosphate, p-toluidene salt*
BI	*Bovine insulin*
BIA	*Biomolecular interaction analysis*
BiP	*Immunoglobulin-binding protein*
bp	*Base pairs*
BSA	*Bovine serum albumin*
°C	*degrees Centigrade*
CDR	*Complementarity determining region*
CIS	*Central immune system*
CLL	*Chronic lymphocytic leukaemia*
Con A	*Concanavalin A*
Da	*Dalton*
dH_2O	*Distilled water*
DHBSS	*Dissection Hank's balanced salt solution*
DMEM	*Dulbecco's modification of Eagle's medium*
DMSO	*Dimethyl sulphoxide*
DNM	*Dominant negative mutants*
DNP	*Dinitrophenol*
dNTP	*Deoxynucleotidyl triphosphates*
dTT	*Dithiothreitol*
EBV	*Epstein barr virus*
EDC	*Ethylenediamine carbodiimide*
EDTA	*Ethylenediamine tetra-acetic acid*

EGTA	*Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid*
ELISA	*Enzyme-linked immunosorbent assay*
ER	*Endoplasmic reticulum*
Fab	*Antigen binding fragment*
$F(ab')_2$	*Bivalent antigen binding fragment*
FACS	*Fluorescence-activated cell sorter*
Fc	*Crystalisable fragment*
FCA	*Freund's complete adjuvant*
FCS	*Fetal calf serum*
FITC	*Fluorescein isothiocyanate*
Fv	*Variable fragment*
GIgG	*Goat IgG*
GSE	*Genetic suppressor elements*
HIV	*Human immunodeficiency virus*
HPLC	*High pressure liquid chromatography*
IG	*Intergenic region*
Ig	*Immunoglobulin*
IMEM	*Iscove's modification of Eagle's medium*
IPTG	*Isopropyl β-D-thiogalactopyranoside*
IVI	*In vitro immunisation*
KLH	*Keyhole Limpet haemocyanin*
L	*Litre*
LOD	*Limit of detection*
LPS	*Lipopolysaccharide*
M	*Molarity*
mA	*Milliamps*
Mab	*Monoclonal antibody*
2-ME	*2-mercaptoethanol*
MLR	*Mixed-lymphocyte reaction*
M_w	*Molecular weight*
NBT	*Nitroblue tetrazolium*
NEAA	*Non-essential amino acids*

NHS	*N-hydroxysuccinimide*
NIP	*4-hydroxy-5-iodo-3-nitrophenylacetyl*
NLS	*Nuclear localisation sequence*
OD	*Optical density*
7-OHC	*7-Hydroxycoumarin*
ORF	*Open reading frame*
PAGE	*Polyacrylamide gel electrophoresis*
PBS	*Phosphate-buffered saline*
PC	*Phosphoryl choline*
PCR	*Polymerase chain reaction*
PEG	*Polyethylene glycol*
PHA	*Phytohaemagglutinin*
PIS	*Peripheral immune system*
PMA	*Phorbol 12-myristate 13-acetate*
PMSF	*Phenyl methyl sulphonyl fluoride*
pNPP	*para-nitrophenyl phosphate*
PWM	*Pokeweed mitogen*
RS	*Rabbit serum*
RT	*Room temperature*
RU	*Response unit*
ScFv	*Single-chain Fv*
SDS	*Sodium dodecyl sulphate*
ssDNA	*Single-stranded DNA*
TCM	*Thymocyte-conditioned media*
TEMED	*N,N,N',N'-tetramethylethylenediamine*
Thyr.	*Thyroglobulin*
TLCK	*Nα-p-tosyl-l-lysine chloromethyl ketone*
Tris	*Tris(hydroxymethyl)aminomethane*
u	*Unit of activity*
UPH$_2$O	*Ultrapure water*
V	*Volts*

ABSTRACT

The suitability of *in vitro* immunisation, for monoclonal antibody (Mab) production, was investigated. A panel of nine Mabs were produced, and analysed for polyreactivity. Three different assays for polyreactivity were utilised, including measurement of the affinity of each Mab for a small panel of antigens. Using these three assays, eight of the nine Mabs were demonstrably polyreactive. It was concluded, that *in vitro* immunisation may not be suitable for Mab production.

A second area of interest was the generation of intrabody libraries, for functional genomic analyses. A PCR-based strategy was developed to convert any ScFv gene, from the Nissim library, to an intrabody gene. As a model, an anti-NIP ScFv gene was converted to an intrabody gene. Cytochemical studies indicated correct cytoplasmic localisation of this intrabody, but poor expression levels.

The strategy was applied to generate a large intrabody library. A novel PCR-cloning methodology was used. This utilised T4 DNA polymerase, and required the creation of a novel vector, referred to as pMIK. The cloning strategy proved very successful and a 'one-shot' intrabody library of $1.3x10^7$ independent clones was generated.

These studies demonstrate the feasibility of the strategy, but suggest that further studies are required to improve intrabody stability.

CHAPTER 1

INTRODUCTION

In 1890, von Behring and Kitasanto demonstrated, that immunisation with diptheria and tetanus toxins, resulted in the production, in the serum, of a soluble substance, capable of neutralising the toxins. They coined the term antibody for this substance and ever since, its function has been inextricably linked to vertebrate immunity, specifically, acquired immunity.

The pre-eminent theoretical framework, used to explain the role of antibody in acquired immunity, is the clonal selection theory, articulated in the 1950's by, among others, Burnet, Talmage and Lederberg (Talmage, 1995). This theory was based on the numerous physicochemical observations on antibodies, since their discovery, and also on a number of *ad hoc* postulates. Briefly, antibody was not one chemically defined substance. Rather, it was a class of heterogeneous, and yet, structurally and functionally related proteins. These proteins were largely identical but individual species varied, primarily, at one structure referred to as a receptor or binding site. **Individual binding sites were chemically defined and allowed that species of antibody to bind with *great specificity* to complementary molecular structures. It was this specificity, which, for the purposes of immunity, required the body to produce a heterogeneous population of binding sites.** Antibodies were expressed on or secreted from B cells, and **individual B cells were restricted to the production of only one species of binding site (referred to as a monoclonal binding site)**. Finally, a primary repertoire of antibodies was ***constitutively expressed*** on the surface of B cells. Within this framework, it was postulated that foreign pathogens, which manage to invade the body, are bound, by that fraction of B cells ***constitutively expressing complementary antibody*** on its surface. The selected B cells then respond, by clonally expanding and secreting antibody possessing the same binding site as on its surface. The secreted antibody binds the pathogen and, subsequently, neutralises it. During the course of this immune response, the antibody displayed by the selected B cells mutates; only those B cells expressing antibodies with improved binding characteristics for the antigen survive and secrete the improved antibody. It is primarily this ***induced (acquired) antibody*** that appears in the serum of vertebrates during immune responses.

Antibody fragmentation studies and amino acid sequencing of monoclonal antibodies soon demonstrated, that antibodies vary, primarily, at one

structure, as predicted (Nisonoff, 1995). This structure was then identified by crystallographic studies as the putative binding site (Lesk and Tramontano, 1992). **The postulate that binding sites possess great specificity was justified, by reference to the many immunochemical studies, since before the turn of the century, on *antisera from animals undergoing immune responses*** (Silverstein, 1989). Formal proof was obtained in 1976, that B cells express one species of binding site only (Nossal, 1995), although the theory had found almost complete acceptance well before this date. Finally, genetic analyses, carried out in the late 1970's and early 1980's (Tonegawa, 1995), demonstrated that humans can produce, constitutively, up to $1x10^{10}$ different binding sites.

That the phrase 'specific antibody' is almost considered a tautology in most textbooks, is due in part to the dominance of this clonal selection theory and the immunochemical observations underlying it. This perception has lead to the widespread use of monoclonal antibody binding sites (antibodies or related molecules) in many scientific disciplines unrelated to immunology. The specificity of antibodies is invaluable in many analytical tools such as enzyme-linked immunosorbent assays (ELISAs), allowing the quantification of ligands such as pesticides, in complex biological matrices, such as foodstuffs. The clinician can also take advantage of monoclonal antibody specificity. In total, 30% of all recombinant proteins currently undergoing clinical trials, are antibodies or related molecules. One concept, frequently described, is the destruction of a tumour, by using a specific antibody to target a toxin to the tumour.

However, immunochemical studies carried out over the past 15 years call into question the established dogma of antibody specificity. **These more recent studies do not dispute earlier studies on the specificity of antibodies *induced during in vivo immune responses*. What they do suggest, is that *constitutively expressed* antibodies can show a polyspecific binding pattern**. It is the primary aim of this chapter, to review these observations (Section 1.2). These observations are of interest, not only to theoretical immunologists, but also to anyone using or producing monoclonal antibodies, due to the fact, that they suggest that the mode of production of monoclonal antibodies could affect their specificity. It is with this in mind, that section 1.3 reviews the production of

these important reagents. In section 1.4, a novel use of monoclonal antibodies is introduced. This is their ectopic expression for phenotypic knockout. This chapter starts, however, with a general introduction to the production and structure of antibodies and derivatives thereof, particularly as it relates to their ability to bind antigen.

1.1 THE PRODUCTION, STRUCTURE AND INTERECTION, OF ANTIBODIES AND DERIVATIVES, WITH ANTIGENS

1.1.1 Prototypic antibody structure: A diagram of a typical antibody, an immunoglobulin G (IgG) molecule, is shown in Fig. 1.1. It is composed of four polypeptide chains, with two identical heavy chains and two identical light chains. The heavy and light chains of this molecule are held together by interchain disulphide bonds and also by non-covalent interactions. The heavy chain consists of four domains named from the N-terminus as V_H, C_H1, C_H2 and C_H3. V stands for variable domain and C for constant domain. The domains are referred to as variable or constant depending on the amino acid sequence variation among individual antibody molecules. The light chain consists in the main of two domains named, from the N-terminus, as V_L and C_L. Between the C_H1 and C_H2 domains lies the hinge region, which allows the two arms of the Y shaped molecule a certain degree of flexible independent motion.

All of the above domains show considerable structural similarity. They consist of two stacked layers of β sheets surrounding an internal space filled with hydrophobic amino acid side chains (Bradbury, 1997) (see Fig. 1.2). A very important feature of the domains is the intrachain disulphide bond which links one β sheet to the second β sheet and stabilizes the entire domain structure.

The antibody binding site is formed by the juxtaposition of one V_H domain with one V_L domain. Thus, each IgG contains two antibody binding sites. The antibody binding site allows the antibody molecule ***to bind different molecular structures (referred to as antigens) selectively and potentially with great specificity. It is the sequence variation in the variable domains with consequent variation of binding site structure that underlies the ability of different antibody molecules to bind to different antigens.*** The binding site structure is discussed more fully in section 1.1.1.3 and its interaction with antigens is the subject of section 1.1.4.

The constant domains (the sum of which is referred to as the constant region), play no part in the binding activity of the antibody although they have a variety of biological activities, which will not be discussed. The interested reader is referred to Elgert (1996).

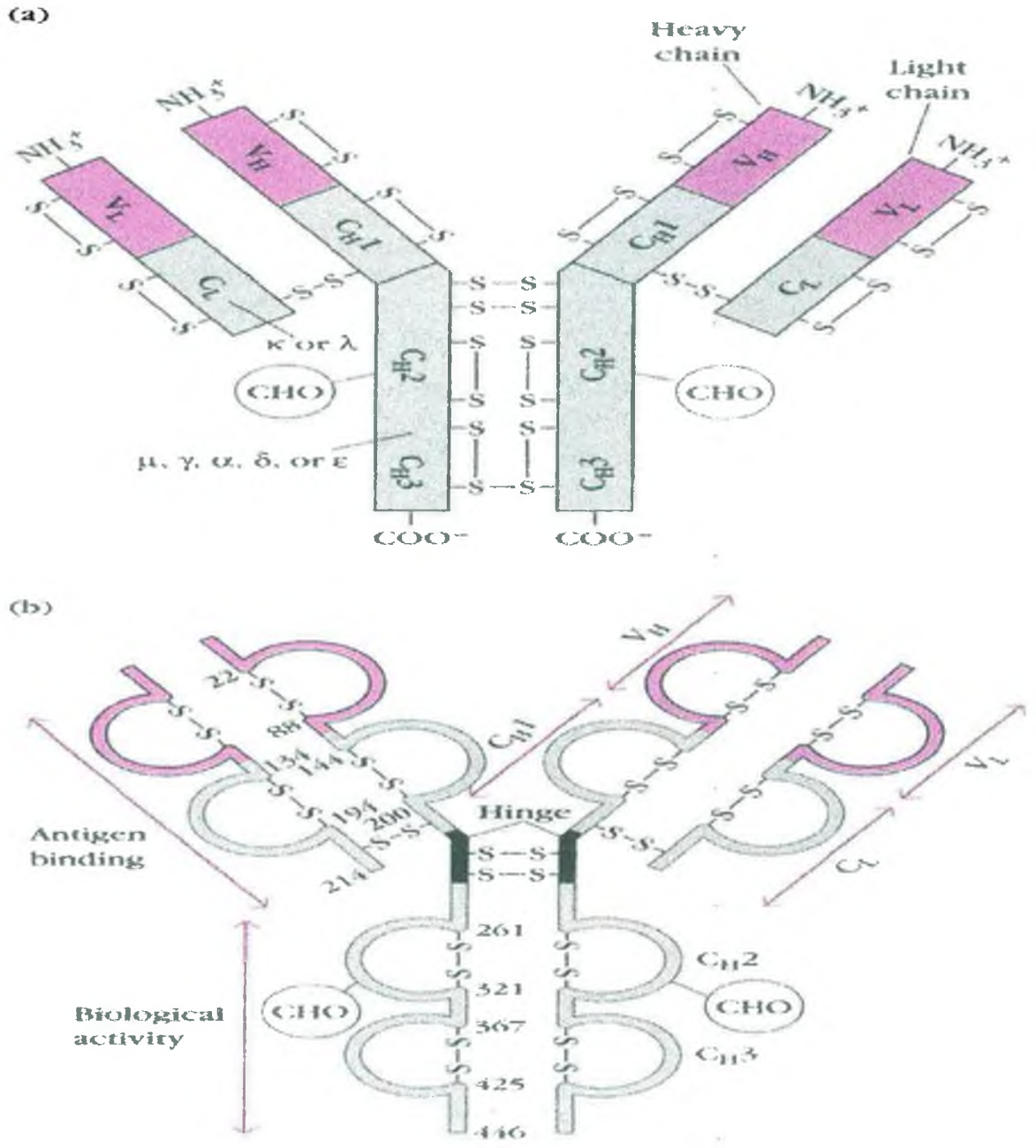


Fig. 1.1: *A schematic of a typical IgG molecule. CHO indicates glycosylation. The intrachain disulphide bonds, which stabilise each immunoglobulin fold, are clearly visible. Also depicted are the interchain disulphide bonds, which covalently link the heavy and light chains. The greek symbols μ, γ, α, δ, and ε refer to the 5 different variants of the heavy chain constant region and the symbols κ and λ refer to different forms of the light chain constant region (see section 1.1.1.1). Diagram reproduced from the internet (http://www.mcms-dal.ca/) with the artists permission.*

*1.1.1.1 **Antibody heterogeneity**:* The antibody molecule is the most variable protein expressed in vertebrates. As discussed by Elgert (1996), there are three types of variation that occur in the antibody molecule.

Isotypic variation: Isotypic variation is a reflection of the expression, by vertebrates, of a limited number of different constant regions. For example humans express five variants of the heavy chain referred to as μ, α, γ, δ and ε. Antibodies containing these heavy chains are referred to as IgM, IgA, IgG, IgD and IgE, respectively. Four different subclasses of the γ chain exist referred to as $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ (IgG_{1-4}). Two different subclasses of the α chain exist, $\alpha 1$ and $\alpha 2$ (IgA_1 and IgA_2). The light chain occurs in two major isotypic variants named κ and λ but there are four λ isotypic variants, λ_1, λ_2, λ_3 and λ_6. Each different constant region is encoded for in the germline of all members of the species by separate gene segments (see section 1.1.2).

Allotypic variation: This is a reflection of limited polymorphism in the constant region gene segments.

Idiotypic variation: This is the primary type of variation in antibodies. It has already been alluded to, and is due to the huge variety of variable domains that can be expressed. In other words idiotypic variability is a consequence of binding site diversity. Humans, for example, can constitutively produce approximately $1x10^{10}$ different binding sites (see section 1.1.2).

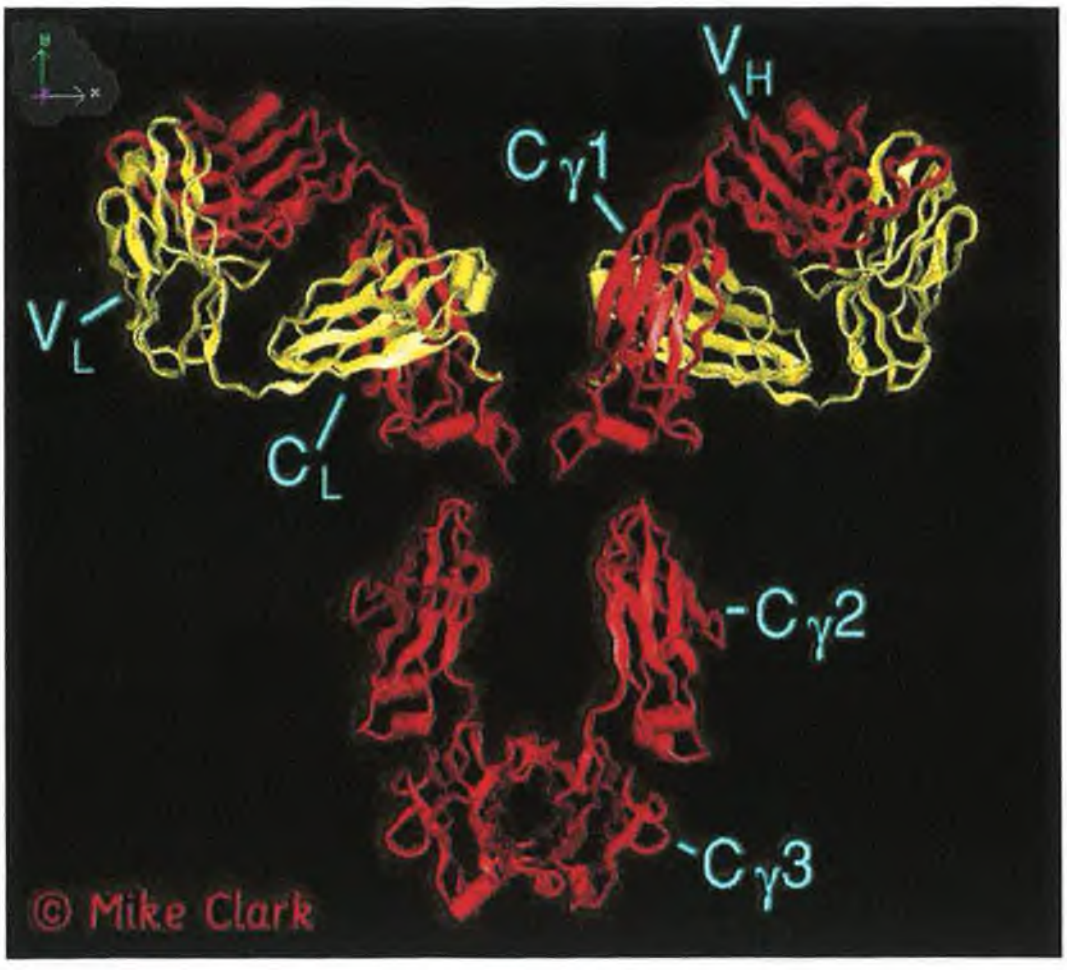


Fig. 1.2: *Ribbon diagram of a typical IgG. The structural similarity between all domains, along with the β sheets, are clearly visible. This diagram is available on the internet at http://www.path.cam.ac.uk/ and is reproduced here with the permission of the artist.*

1.1.1.2 ***Dimeric and polymeric antibody molecules*:** The secreted IgM molecule exists in pentameric form. The five monomeric units are arranged radially with the variable domains pointing outwards and the constant region inwards (Elgert, 1996). They are connected to each other by disulfide bonds mediated by a cysteine residue at the C-terminus of the μ chain. This pentameric IgM molecule also has a protein, referred to as the J chain, associated with it. This chain appears to restrict polymerisation to pentamers, as opposed to higher polymers (Bradbury, 1997).

Similarly, IgA exists primarily in dimeric form with an associated J chain and another protein referred to as the secretory component which serves to

protect the dimer from proteolytic cleavage. The precise alignment of these proteins in the polymer is unknown (Elgert, 1996).

*1.1.1.3 **Antibody binding sites***: The huge variety of antibody binding sites that the body can produce might suggest that antibody binding sites are generated in a chaotic and random fashion. This is not so. On the contrary, at the protein level, crystallographic analyses have indicated that there is a substantial degree of rigor to the way the binding site is composed. This is mirrored by the orderly fashion in which the vast diversity of antibody binding sites is encoded in the genome (see section 1.1.2)

Large-scale amino acid sequencing of monoclonal antibodies gave some of the first clues to the structure of the antibody binding site. Wu and Kabat (1970) on comparing these sequences noted that variability was most pronounced in three regions of the variable sequence of the heavy and light chains, these regions being separated by the so-called framework sequences. These regions were referred to as hypervariable regions or complementarity determining regions (CDRs). They were named from the N-terminus (H for heavy, L for light) as H1, H2, H3, and L1, L2 and L3. Wu and Kabat (1970) predicted that upon analysis of the quaternary structure of the antibody molecule these regions would be found juxtaposed and would form the antibody binding site. This prediction was borne out upon analysis of the three-dimensional structure of a number of antibodies and complexes thereof (Lesk and Tramontano, 1992).

Like all other antibody domains, variable domains all contain a double β-sheet motif. This structure is formed by the framework sequences. Each double β-sheet has associated with it three loops which connect its strands. The loops correspond to the CDRs. This correspondence is not absolute and there are differences between the definitions of the CDRs based on primary sequence and based on the three dimensional structure (MacCallum *et al.*, 1996). Four of these loops (CDRs1-2) are hairpin loops; i.e. they connect successive strands of the anti-parallel β-sheet. The remaining two connect the two β-sheets of their respective domains (Lesk and Tramontano, 1992). All loops are exposed on top of the variable domains and come together to form the antibody binding site

(Fig. 1.3). Thus, it has been said that the framework regions provide a scaffold upon which the antibody binding site is constructed (Lesk and Tramontano, 1992).

Chothia and Lesk have analysed the loop structures on all antibodies, whose three-dimensional structures have been solved, and have noted a further

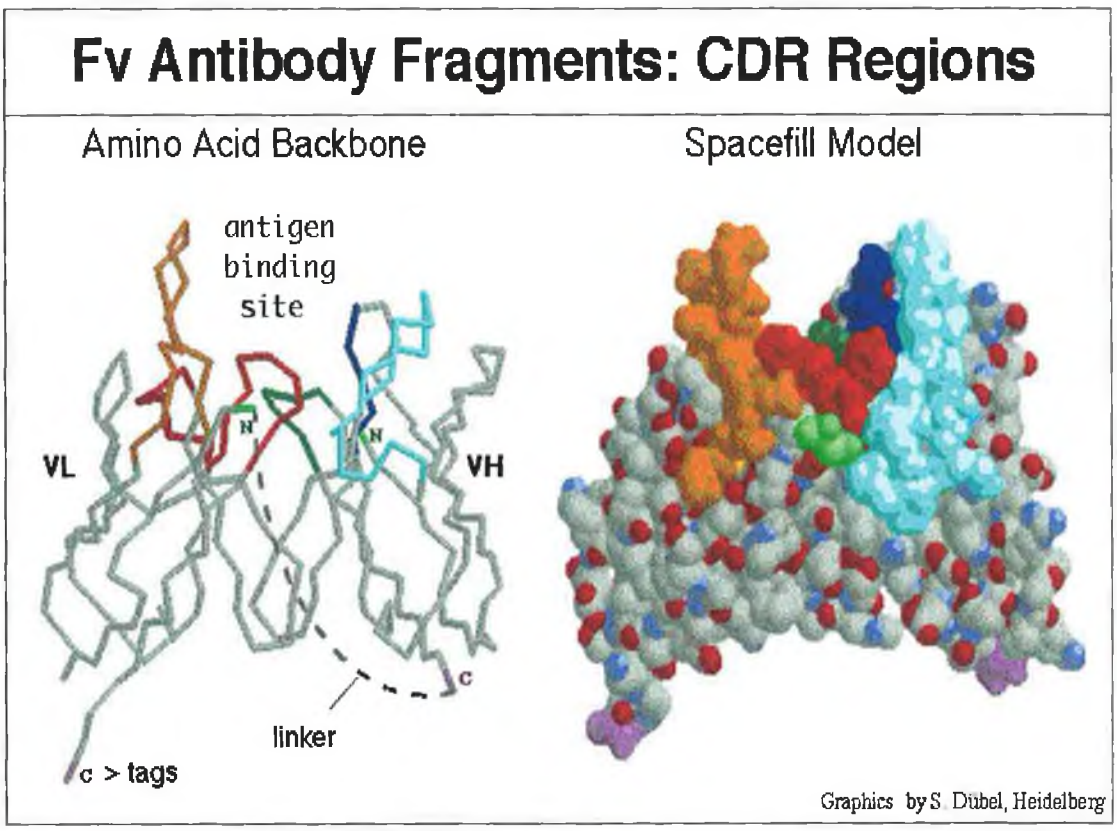


Fig. 1.3: *Colour coded diagram showing the 6 CDRs of a Fv (see section1.1.3) molecule. Note also the dotted line indicating where the linker segment would be in an ScFv (see section 1.1.3) molecule. The space filling diagram clearly shows how the juxtaposition of the CDRs forms a ridged binding site (in this case). This diagram was obtained from the internet (http://www.mgen.uni-heidelberg.de/) and is reproduced here with the artists permission.*

level of order to binding site construction (Lesk and Tramontano, 1992). They have observed that five of the six loops (the exception being H3) can each take on only a limited number of main chain conformations or "canonical structures" (Chothia *et al.*, 1989). Thus L1 loops have four basic canonical structures, L2 one, L3 three, H1 two and H2 four (Lesk and Tramontano, 1992). Whether one canonical structure is chosen over another is determined primarily by the length of the loop, and the presence of particular residues at certain sites in the loop. These sites are relatively conserved in the hypervariable regions (Lesk and Tramontano, 1992). For example the presence in position 90 of a polar side-chain and a proline at position 95 determines one particular canonical structure for L3. The remaining residues in the hypervariable loops are free to vary and do not greatly affect the main-chain conformation. However, their different side-chains determine the surface topography of the binding site along with the charge distribution and other physiochemical characteristics. (Lesk and Tramontano, 1992). It is also worth noting that the determinants of canonical structure do not reside exclusively in the loops themselves. Rather the framework regions do not solely act as a scaffold for the loops but residues within it can affect loop conformation. For example, the size of the residue at position 71, a site in the double β-sheet motif of V_H, affects the conformation and position of H2 (Lesk and Tramontano, 1992).

Even an analysis of the overall topography of antibody binding sites of known three-dimensional structure has indicated some degree of "restriction" or order. Using an objective fractal measure, MacCallum *et al.* (1996) showed that binding site topographies all segregate in one of four classes: concave, moderately concave, ridged and planar. These classes also correlate quite well with antigen type. Thus concave and moderately concave are associated with binding of haptens, ridged with peptides, DNA and carbohydrates and planar is associated with protein antigens (MacCallum *et al.*, 1996).

Finally most of the observations noted above have been based on crystallographic analyses and assume that antibodies are stable entities. **Nonetheless, observations of antibodies in solution indicate that antibodies may exist in at least two structural forms in dynamic equilibrium (Milstein and Neuberger, 1996).**

1.1.2 **Genetics of binding site diversity:** The generation of binding site diversity varies between species (Milstein and Neuberger, 1996) and this discussion focuses on humans and mice who appear to utilise similar mechanisms. Somatic events are the primary generators of diversity. The primary (naïve) repertoire, of antibody binding sites, is generated at the pre-B cell stage of lymphopoiesis, by a process known as recombination (Milstein and Neuberger, 1996). **This repertoire, in the terminology of some authors (see for example Coutinho, 1989), is referred to as preimmune, to indicate that it is constitutively expressed on B cells in the absence of any *in vivo* immune response.**

In the germline of each individual, families of gene fragments exist in multiple non-identical copies. For example the human immunoglobulin heavy chain gene locus is located at band q32, of the long arm of chromosome 14 (Kipps, 1997). It contains 50 functional heavy chain variable region gene segments (V_H), at least 24 diversity (D) gene segments and 6 functional J_H gene segments (See Fig. 1.4). The light chain gene loci (kappa on chromosome 2 and lambda on chromosome 22) are similarly arranged, containing multiple V_L and J_L gene segments. Recombination of one element from each of the V_H, D and J_H families creates an exon encoding the V_H domain and, similarly, recombination between V_L and J_L fragments creates an exon encoding the V_L domain (Milstein, 1990; Milstein and Neuberger, 1996). The V fragments encode the CDRs 1-2 and the junctional regions (V(D)J or VJ) encode CDRs 3.

Of note, this process primarily generates diversity in CDR3 (in particular H3) and there is only limited diversity in CDRs1-2 (Table 1.1) (Milstein and Neuberger, 1996). The greater diversity in CDR3 is not solely due to the greater number of elements that recombine to encode it. It is also dependent to a major extent on the imprecision in the joining mechanism (Milstein and Neuberger, 1996). In the case of H3 it is also dependent to a large extent on the addition of extra bases (N) at the VD and DJ boundary during joining (Milstein and Neuberger, 1996).

Overall, a picture has emerged of a potential preimmune repertoire of at least $1x10^{10}$ different antibody binding sites encoded in the germline. It should be noted that the generation of the preimmune repertoire is a quasi-random (not completely random) process for a number of reasons.

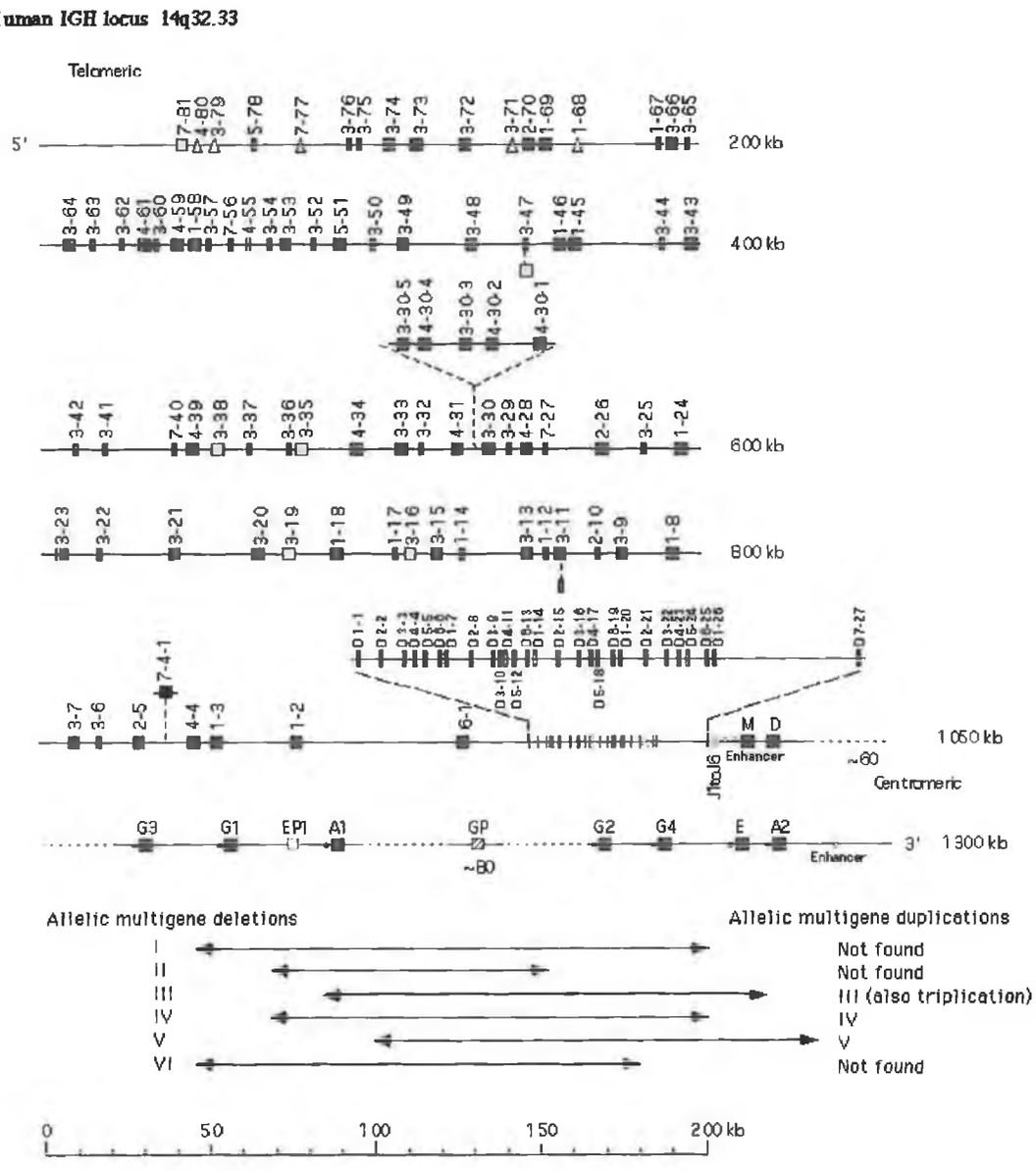


Fig. 1.4: *The structure of the immunoglobulin heavy chain locus, is shown above. Green boxes represent functional V_H gene segments. Yellow boxes represent ORFs. Red boxes represent pseudogenes. Navy boxes represent D segments. The J segments are represented by bright yellow boxes. The constant region gene segments are shown in blue. This diagram is reproduced here with the permission of: "IMGT, the international ImMunoGeneTics database http://imgt.cnusc.fr8104 (Coordinator: Marie-Paule Lefranc, Montpellier, France lefranc@ligm.igh.cnrs.fr) For reference: Nucleic Acids Research, 25, 206-211 (1997)"*

Firstly, the fragments carried in the germline are conserved and reflect the evolutionary gene history of the species and Darwinian selection (Milstein, 1990). Secondly, the recombination process itself is not a random one and some combinations are found with much greater frequency than others (Milstein and Neuberger, 1996).

Thus, upon initial challenge by a pathogen, the individual relies on the chance recognition of the pathogen, by antibodies of the preimmune repertoire on the surface of B cells. Antibodies are selected from this repertoire based on their frequency and affinity for antigen (Milstein and Neuberger, 1996). This initial response is referred to as a primary response. However, following this initial chance encounter the individual "learns" or "adapts" to recognise the antigen. This "learning" process takes place in the microenvironment of the germinal center. In this microenvironment Darwinian selection for antibodies with improved binding characteristics takes place (Milstein, 1990; Milstein and Neuberger, 1996). In general, the result of this selection is the generation of antibodies with increased affinity for the antigen. Hence, this process is referred to as affinity maturation. This selection is wholly dependent upon and must be preceded by mutation of antibody genes to provide a population of binding sites from which to select the best. A specific mutation mechanism has evolved to accomplish this. It is referred to as somatic hypermutation and is the second generator of diversity.

A limited number of B cells selected by the antigen from the preimmune repertoire enter the germinal center and undergo rapid pauci-clonal proliferation. During this stage of B cell development the rearranged V(D)J and VJ loci are subject to a high rate of mutation ($1x10^{-3}$ mutations per base per division) (Milstein and Neuberger, 1996). The mechanism of somatic hypermutation is uncertain but may rely on Topoisomerase II to create single-strand nicks followed by the recruitment of error-prone DNA-repair mechanisms (Milstein and Neuberger, 1996). Regardless of the mechanism, the net result is the generation of a population of daughter cells derived from antigen-selected preimmune B cells but expressing antibody of widely diverging affinity for the selecting antigen on their surface. Within the germinal center it is considered that antigen is trapped in limiting amounts on the surface of specialised cells referred to as follicular dendritic cells (Milstein and Neuberger, 1996).

Parameter	Diversity of CDR1 and CDR2
v germline genes	100 (V_κ and V_λ) x 50 V_H
combinatorial	
junctional diversity	
total	**$5x10^3$**

Diversity of CDR3 (heavy chain)	Diversity of CDR3 (light chain)
50 VH x 4 JH x 20 D	(50 V_κ x 4 J_κ) + (50 V_λ x 4 J_λ)
x 6 (VD), x 6 (DJ), x 50 (N segments)	x 3
$1x10^7$	**$1x10^3$**

Table 1.1: *A simple mathematical model allowing a calculation of the total antibody diversity generated by somatic recombination is presented. The total diversity due to H1, H2, L1 and L2 is calculated based on the assumption that any of the 100 V_κ or V_λ gene segments can be paired with any V_H gene segment. The diversity due to H3 and L3 are considered separately from H1, H2, L1 and L2, as they are generated in a different fashion (recombination and junctional diversity). For both H3 and L3, this mathematical treatment assumes that all V, D (if applicable) and J segments are equally permitted to recombine with each other. To take into account, junctional diversities, multiplication factors are provided (taken from Milstein and Neuberger, 1996). When these are used, to multiply the diversity due to recombination, the total diversity due to L3 becomes $1x10^3$. The total diversity due to H3 is $1x10^7$. To calculate the total diversity due to somatic recombination, it is assumed that each L3 is equally likely to pair with a given H3. This gives a total antibody diversity of $1x10^{10}$. The diversity due to H1, H2, L1 and L2 is not taken into this calculation. This is because, diversity in these loops, being dependent on V gene usage, is not generated independently of the diversity in L3 and H3.*

Under these limiting conditions cells expressing antibody of highest affinity are at a selective advantage with regard to binding of trapped antigen. Failure to bind antigen results in apoptosis, whereas those B cells that bind antigen are programmed for survival, emerge from the germinal center and become memory cells and plasma cells (Milstein and Neuberger, 1996). **This stage of the immune response is referred to as secondary and the antibodies are referred to as secondary antibodies. They can also be referred to as immune antibodies to indicate that their appearance is wholly dependent upon an *in vivo* immune response.** The combination of somatic recombination and hypermutation results in almost unimaginable antibody diversity with figures such as $1x10^{30}$ different antibody binding sites being mentioned in the literature (Milstein and Neuberger, 1996).

In many important respects hypermutation and recombination are two complementary mechanisms. Thus, somatic recombination results in a diverse population of antibody binding sites, selected in a Darwinian fashion over many hundreds of thousands of years, and reflects the evolutionary history of the species. Somatic hypermutation results in the generation of a diverse population of binding sites, selected in a Darwinian fashion but over the course of a couple of days, and reflects the history of the individual. Also, somatic hypermutation is a quasi-random process; even in the absence of the bias induced by antigen selection certain residues in the variable region appear to be "hot-spots" for mutation (Neuberger and Milstein, 1995). At the level of CDRs, CDR1 appears to be mutated with the greatest frequency, followed by CDR2 and CDR3 (Milstein and Neuberger, 1996). This is the exact opposite of the pattern of variability created by recombination. Indeed, there is as much variability in the framework residues in the human germline as there is in CDR1 (Huston *et al.*, 1996). Therefore, hypermutation complements recombination in that it spreads diversity to apical regions of the binding site (Huston *et al.*, 1996). The net effect appears to be fine-tuning of the binding site topography, or indeed apical residues in the binding site making contact with the antigen (Milstein and Neuberger, 1996).

1.1.3 Antibody derivatives: The antibody domain structure has greatly facilitated the production, by chemical and genetic means, of a variety of antibody derivatives with binding activity similar to the parental antibody molecule.

1.1.3.1 Chemical methods: Fab fragments are generated by digesting whole IgG with papain. Papain digests in the hinge region just above the double interchain disulfide bonds (see Fig. 1.1). Fab fragments contain the V_H and C_H1 domains from the heavy chain and the entire light chain. The chains are linked by a disulfide bond and the resulting monovalent fragment maintains binding activity (Nisonoff, 1995).

$F(ab')_2$ fragments result from the digestion of whole IgG with pepsin. Pepsin digests at a similar position to papain but results in a bivalent fragment (Nisonoff, 1995). This is because it digests in the hinge region, but just below the two disulfide bonds.

These two antibody derivatives may be useful in certain circumstances. This might be the case for the clinical use of antibodies. If an anti-tumour antibody was to be used clinically, a smaller molecule would presumably be advantageous in so far as it would have better tumour penetration characteristics. Also the Fc portion of IgG can lead to non-specific binding of whole IgG.

1.1.3.2 Genetic methods: It is possible to generate genes encoding antibody heavy and light chains. Insertion of these genes into a suitable vector allows functional expression, of the antibody or a variety of antibody-like molecules, in a number of hosts. Much of this technology has been made possible by the elucidation of the structure of the genetic loci encoding antibody heavy and light chains in the late 70's and early 80's (see section 1.1.2). Furthermore, this technology has been greatly facilitated by PCR.

Although functional antibody and fragments can be expressed in myeloma cells (Morrison, 1985) and yeast (Cabilly *et al.*, 1984), by far the most common host for production is *E. coli*. This is due primarily to the ease with which it can undergo genetic manipulation and fermentation.

Initial work expressing whole heavy and light chains in *E. coli* was not promising. Heavy and light chains formed intracellular inclusion bodies with no activity (Wood *et al.*, 1985). Activity could be restored by denaturation of the

inclusion bodies followed by refolding *in vitro*. Lack of expression of functional antibody was not too surprising. Antibody folding and assembly in the endoplasmic reticulum (ER) of lymphoid cells is a complex process involving disulfide bond formation and requiring many chaperone proteins. The ectopic environment of *E.coli* may not provide a suitable environment for correct antibody folding and assembly (Wood *et al.*, 1985).

Two papers, demonstrating the feasibility of functional expression of antibody fragments in *E. coli*, appeared in 1988. Skerra and Pluckthun (1988) approached the problem by attempting to express synthetic genes encoding the V_H and V_L domains from the myeloma protein McPC603 in *E. coli*, while translocating the resulting nascent polypeptides into the periplasm. Heterodimerisation of correctly folded domains was expected to produce a monovalent antibody fragment, referred to as a Fv, with binding activity.

The rationale for this approach was twofold. Firstly, a Fv fragment should have reduced problems with incorrect folding and assembly and, secondly, translocation into the periplasm of the chains might more closely resemble transport of polypeptides across the ER membrane. Synthetic genes encoding the V_H and V_L domains were precisely fused to the *ompA* and *phoA* signal sequences, respectively, in a dicistronic operon and expressed from a suitable vector. Fully functional soluble Fv accumulated in the periplasm and showed the same affinity as the parental myeloma protein (Skerra and Pluckthun, 1988).

Using a similar approach Better *et al.* (1988) fused the leader sequence from *pelB* to a gene encoding the V_H and C_H1 domains of an antibody and also to a separate gene encoding the corresponding light chain of the antibody. The resulting genes were cloned into a vector as a dicistronic operon and functional Fab was excreted into the culture supernatant.

Fv fragments unfortunately are of limited stability at low protein concentrations and under physiological conditions. A number of strategies have been published to increase their stability (Glockshuber *et al.*, 1990). The most popular strategy has been to create an artificial antibody derivative referred to as a single-chain Fv (ScFv). Huston *et al.* (1988) constructed a synthetic gene containing the coding sequence for the V_H domain of an antibody and also the coding sequence for its V_L partner. Connecting the 3' end of the V_H domain to

the 5' end of the V_L domain was a linker sequence encoding (gly-gly-gly-gly-ser)$_3$. Thus the V_H and V_L domains are both on the one polypeptide chain. The 15 amino acid linker was chosen because it is just long enough to bridge the gap between the C-terminus of the V_H domain and the N-terminus of the V_L domain and also because it should not form any secondary structure. The resulting ScFv had an association constant for digoxin about six times smaller than a Fab fragment of the parental antibody.

All the above mentioned reports cloned genes or gene fragments using techniques such as classical cDNA cloning from libraries using constant region gene probes followed by site-directed mutagenesis, amino acid sequencing of heavy and light antibody chains followed by solid phase synthesis of the complementary genes, to name but two. These techniques are almost invariably successful but take a long time. The application of PCR has resulted in a much shorter time frame for cloning antibody genes and gene fragments. However, there are potentially a number of problems inherent to cloning antibody genes from hybridomas by PCR and these are well discussed by Bradbury (1997).

Orlandi *et al.* (1989) were the first to clone antibody genes using PCR. Although V_H, V_L, J_H and J_L gene segments are variable they do contain some relatively conserved regions particularly at the 5' ends of V_H and V_L gene segments and the 3' ends of J_H and J_L gene segments. Thus Orlandi *et al.* (1989) constructed "universal" primers to clone genes encoding V_H and V_L domains from hybridomas by reverse transcriptase PCR. The resulting genes were cloned into special vectors allowing them to be expressed as part of a full antibody heavy chain and a full antibody light chain, respectively. NSO double transfectants secreted full antibodies with binding activity.

Variable regions cloned in this fashion are now normally assembled in the form of a ScFv or a Fab and expressed in *E. coli*. Genes encoding Fabs are generated using 3' primers recognising the 3' sequence of the C_H1 or C_L domains, (Bradbury, 1997).

Two other antibody fragments have been found to possess binding activity. Ward *et al.* (1989) found that V_H domains expressed on their own had binding activity. Unfortunately these fragments have low solubilities, which has hindered their usage. Even individual CDRs have been explored as potential binding molecules (Levi *et al.*, 1993).

1.1.4 Antibody-antigen interactions: Antibodies and antigens interact solely at the antibody binding site. Interactions are characterised by a high degree of complementarity (surface topography, charge etc..) between the surfaces at the interface (Wilson and Stanfield, 1993). **It is this requirement for complementarity that underlies the ability of monoclonal antibodies to selectively bind certain antigens only. It does not imply great specificity, however, as will be discussed in section 1.2. Furthermore, although antibody-antigen interactions have often been explained in terms of a lock and key mechanism (as befits the concept of specificity), an induced-fit mechanism may be more appropriate as indicated by analyses of antibodies in both free and complexed forms (Wilson and Stanfield, 1993). In fact rather large conformational changes can take place in the antibody binding site including changes in gross topography (MacCallum *et al.*, 1996).**

Analyses of the contribution of individual CDRs to binding indicates that at least four CDRs are involved in each case analysed so far, although it is also true that all six are never involved (Wilson and Stanfield, 1993). L3 and H3 tend to predominate in binding interactions (MacCallum *et al.*, 1996) and conversely L2 only rarely engages in antibody-antigen interactions (Wilson and Stanfield, 1993; MacCallum *et al.*, 1996).

Examination of the contribution of individual residues to binding have also been carried out (MacCallum *et al.*, 1996). **On average only 28% of residues in the CDRs form contacts with antigen in all of the complexes whose structures have been determined so far (MacCallum *et al.*, 1996).** Results indicate that the propensity of a residue in a CDR to form a contact with the antigen correlates with its position in the binding site. Those residues closest to the center have the greatest chance of interacting with an antigen. The "binding epicenter" (MacCallum *et al.*, 1996) is slightly displaced towards H3. Residues apical to the binding site interact less frequently and then only with larger antigens. The results of MacCallum *et al.*, (1996) also suggest that residues outside the conventionally defined CDRs (i.e. in the framework regions) can be important to antibody-antigen interactions when they are solvent accessible. Indeed, they have proposed a new classification for CDRs based on their contact analysis.

1.2 ANTIBODY POLYSPECIFICITY

1.2.1 Historical and theoretical background: The absolute specificity of the antibody-antigen interaction was an integral part of Erhlich's influential side-chain theory of antibody formation (Silverstein, 1989). This concept of absolute specificity was partly based on Fischer's work on the specificity of enzyme-substrate reactions and also immunochemical studies of antisera. It was also based on the philosophical viewpoint that nature was discontinuous and that biological interactions were qualitatively absolute (Silverstein, 1989). **This theory also suggested a structural basis for this absolute specificity. It was said to be a consequence of the interaction of chemically defined complementary molecular structures (Silverstein, 1989).**

Landsteiner took a diametrically opposite philosophical viewpoint. Antibodies should show a quantitative and continuous gradation of affinity with various antigens (Silverstein, 1989). He studied the immune response to haptens in order to better understand immune cross-reactivity. He demonstrated that appropriate antisera could distinguish between, while at the same time show reactivity against ortho-, meta- and para-substituted azobenzoates (Silverstein, 1989). Landsteiner took this as evidence of cross-reactivity but many others took it as proof of the great specificity of the immune response.

Proof, originating in the 1940s, that an antiserum was composed of a mixture of different antibodies against the antigenic structure, again affected the concept of immunological specificity. As pointed out by Talmage (Silverstein, 1989a), **the fact that a mixture of antibodies showed specificity did not imply that the individual antibody molecules were just as specific.** In fact, as long as each individual antibody showed reactivity with the antigen used to raise the antiserum, they could each have distinct reactivity patterns and the antiserum would still show statistical specificity for that antigen. This theory not only allowed cross-reactivity among similar antigens but among dissimilar antigens.

However, it was primarily the advent of clonal selection theories and, in particular, clonal deletion theories, that affected the concept of antibody specificity. It was Erhlich who coined the phrase 'horror autotoxicus' to indicate that, since antibodies mediate the destruction of the substance they bind to, there

must be some mechanism by which antibodies are prevented from reacting against self-constituents of the vertebrate. The clonal deletion theory stated that the protective mechanism was the deletion of all B cell clones, with specificity towards self, during fetal and neonatal life. **As discussed by Cohn (1992) a highly specific antibody molecule is a prerequisite if deletion of autoreactive clones is a physiological process and is the explanation for the self/ nonself discrimination. This high specificity, in turn, required a very large primary repertoire in order to provide the vertebrate with an adequate degree of protection against the wide variety of pathogens in the environment.** Indeed, there is an immense primary repertoire of antibody binding sites (see Table 1.1).

To summarise, the arguments in favour of a highly specific antibody molecule are three-fold. Immune antisera are highly specific. The structure of antibodies (they are chemically defined) ensures that they cannot be but specific. Finally, their function (they provide acquired immunity against foreign substances without reacting against the self) implies that they must be specific. However, those very characteristics of antibodies (structural and functional) used as the basis of arguments for a specific molecule can equally be used to argue in favour of a polyreactive molecule. For example, the notion that a binding site is chemically defined, and as a result binds with great specificity may be incorrect. Ghosh and Campbell (1986) have pointed out, that if you raise an antibody to an antigen there are a number of possible degrees of interaction. The antigen, conceivably, could avail of all possible contact points in the antibody binding site, and the interaction should be of high affinity. Even if this was so, a similar antigen, which can only make contact with a subset of the available contact points, should still result in an interaction of moderate affinity. If the original antigen only avails of a subset of the available contact points, then a completely dissimilar antigen may bind to the binding site with a moderate affinity using the excess binding sites. It has already been discussed in section 1.1.4 that antibodies, on average, only utilise 28% of their available contact sites when binding to antigens. Other aspects, of binding site structure, which may mitigate against a highly specific molecule, includes the flexibility of the binding site already alluded to (see section 1.1.4) and the fact that antibodies

may exist in a number of different forms, in dynamic equilibrium (section 1.1.1.3).

Biological arguments can also be made in favour of a polyreactive antibody molecule. Current knowledge of the generation of antibody diversity (see section 1.1.2) suggests that the **preimmune repertoire is not sufficiently large for host protection if a highly specific antibody molecule is the rule**. It has been argued, on theoretical grounds, that antibody multispecificity is essential to defend the body against the universe of potential pathogens. Calculations indicate that as much as 10^{16} different antigenic structures may exist in nature (Silverstein, 1989a). As a result, neither man (10^{13}), mouse (10^{9}) or even smaller vertebrates possess enough lymphocytes for protection if absolute specificity is inherent to antibodies (Silverstein, 1989a). In fact, significant multispecificity would appear to be a pre-requisite for survival. The case for absolute specificity is diminished even further when the degeneracy of the immune response is analysed. There may be 5000 different clonotypes in mice capable of reacting with a hapten such as dinitrophenol (DNP). Each clonotype may be present at 10 precursors per mouse (Silverstein, 1989a). Given the number of B cells the mouse possesses there could only be 6000 different antigenic structures against which it can react if absolute specificity is invoked (Silverstein, 1989a).

Similar notions were put forward by Milstein and Neuberger (1996). They noted that clonal selection *per se* does not impose a requirement on the preimmune antibody repertoire, to be either highly specific or of high affinity. **Diversity in the preimmune antibody repertoire, in their view, is required to increase the average affinity of the initial immune response, not as a consequence of the monospecificity of this repertoire.** They argue that specificity and affinity are opposite sides of the same coin, and, with affinity maturation, the immune response becomes increasingly specific for antigen.

How does one counter the argument that clonal deletion imposes a biological pressure for a highly specific antibody molecule? This may be achieved by arguing that clonal deletion is not the sole mechanism by which self-tolerance is maintained. **Two-signal models, as exemplified by Matzingers 'danger hypothesis' (Matzinger, 1994), suggest that the body actually does not have to make a self/non-self discrimination. In recent**

times, Coutinho and colleagues have put forward an immune network theory postulating that the immune system actively recognises self, the so-called 'self-assertion'. All self-reactive antibodies are internally activated and recruited into a highly connected network of self components (including variable regions). This is the so-called 'central immune system (CIS)'. It is inhibited from undergoing large clonal exponential expansions (which characterise conventional immune responses to exogenous antigens and would in this case lead to pathologic autoimmunity) by network dynamics. The remaining resting lymphocytes (constituting the 'peripheral immune system (PIS)') are, however, free to undergo conventional large clonal expansions in response to foreign pathogens along with affinity maturation, as described in section 1.1.2. It is the PIS that gives rise to protective immune responses. This second generation immune network theory is more fully discussed in the following references: (Holmberg and Coutinho, 1986; Coutinho, 1989; Cohen and Young, 1991; Varelo and Coutinho, 1991; Coutinho and Avrameas, 1992; Coutinho, 1995). This theory is not only robust to the existence of polyreactive antibodies, but, establishing and maintaining an immune network ***is absolutely dependent on polyreactive antibodies*** (section 1.2.6).

The final argument for a specific antibody molecule is that immune antisera are highly specific. This fact cannot be contested. Nonetheless, much evidence is now available that preimmune antibodies may be polyreactive in contrast to immune antibodies.

1.2.2 Evidence for multispecificity: Formal proof that monoclonal antibodies can be multispecific was first provided by Richards *et al.* (1975). They showed that a myeloma protein could bind with moderate affinity to two "shelf-selected" chemicals namely DNP-lysine and methyl naphthoquinone. **Much experimental evidence, both direct and indirect, has since accumulated indicating that, in both mice and men, preimmune antibodies can be polyreactive.**

The serum (and indeed bodily secretions such as saliva and colostrum – see Quan *et al.* (1997)) of unimmunised (and antigen-free) mice and humans is reactive with a plethora of antigens both self-antigens (blood-group antigens, organ extracts, polypeptides.) and foreign antigens (bacteria, virus'). These

reactivities are due to antibody referred to as natural antibody as initially it was not considered to be specifically induced by antigen (Avrameas and Ternynck, 1995; Coutinho *et al.*, 1995). More recent evidence suggests that this repertoire of antibodies is the result of a normal T cell dependent primary immune response to self-antigens (Coutinho *et al.*, 1995). From the point of view of idiotype network theory it is a consequence of and evidence for the internal activity of the CIS (Coutinho, 1989). This autoreactivity is referred to as a physiologic autoreactivity to distinguish it from pathologic autoreactivity. Reactivity appears to be unequal over the somatic self and is primarily directed against evolutionarily conserved autoantigens (Coutinho *et al.*, 1995; Dighiero, 1997). From the point of view of this discussion what is important is that much of the analyses of polyreactivity have been carried out on natural antibodies. This work has provided impressive evidence in favour of the concept of polyreactive antibodies.

On passing normal serum through a TNP-lysine immunoadsorbent column, Berneman *et al.* (1992) found that 20% of the total antibody could be eluted from the column. Such a large percentage is indicative of polyreactivity. Many similar findings have been reported in the literature (Avrameas and Ternynck, 1995 and ref. therein; Dighiero, 1997 and ref. therein). The eluted antibody was then used in an immunoblot. Crude protein extracts from a variety of Balb/C organs were separated through a SDS-PAGE gel and electrotransferred to nitro-cellulose. When the eluted antibody was used as a primary probe of this protein template, many protein bands were stained. This again is indicative of polyreactivity. Such observations as these have been extended and strengthened by analyses at the clonal level.

Unimmunised splenocytes have been immortalised by somatic cell fusion and the resulting hybridomas (presumed generated from naturally activated splenocytes) are characteristically polyreactive as indicated by measuring their affinity for a small panel (in general < 10) of randomly chosen antigens (Ternynck and Avrameas, 1986). Indeed, even for proponents of the concept of polyreactivity, the polyreactivity can often be puzzlingly large. Some Mabs show significant affinities for all antigens that they are tested against.

An assay referred to as a chamber ELIspot assay has been developed which allows analysis of B cells at the single cell level for reactivity against a

pair of antigens simultaneously (Klinman *et al.*, 1991; Klinman, 1992). This assay has been used to assay for B cells secreting polyreactive antibody among the naturally activated splenic B cells in mice. For example in Balb/C mice 5-23% of B cells secreting IgM reactive with TNP-KLH could also react with actin or ovalbumin (Klinman, 1994). Similar levels of cross-reactivity were determined using this assay in the naturally expressed repertoire of IgM-secreting B cells in the peripheral blood of humans (Klinman *et al.*, 1994).

Other B cell repertoires have been analysed for polyreactivity. For example, evidence that B cells have the potential to produce polyreactive antibody has been provided by studies of leukaemic cells from sufferers of chronic lymphocytic leukaemia. Analysis by ELISA of antibody produced by cultured leukaemic cells have indicated that they tend to secrete antibody reactive against a broad panel of antigens (Kipps, 1993).

Casali's group has used EBV to polyclonally activate and transform human B cells. The transformed B cells can subsequently be grown, under limiting dilution conditions, to produce large numbers of B cell clones. The resulting population of clones appears to be an unbiased sample of the original infected B cell population (Casali and Notkins, 1989). Using this strategy indirect evidence was obtained that at least some of the B cells in human peripheral blood are polyreactive. Culture supernatants from over 100 monoclonal IgM-secreting B cells were analysed by ELISA for reactivity against a panel of self and foreign antigens. In the case of each antigen tested approximately 3-13% of the clones tested positive (Nakamura *et al.*, 1988; Casali and Notkins, 1989). It was suggested that this could only be explained by the presence of B cells secreting polyreactive antibody in this B cell repertoire. This was formally proved by producing hybridomas from a panel of transformed B cells secreting antibody with rheumatoid factor activity and measuring the affinity of the resulting monoclonal antibodies against a panel of self and foreign antigens. Of 10 IgM secretors tested all showed affinity for more than one of the antigens on the panel (Nakamura *et al.*, 1988).

All the repertoires referred to above are preimmune repertoires. With regard to immune antibodies (antibodies associated with an ongoing *in vivo* immune response), it appears to be a generally held view that they are monospecific (e.g. Bouvet and Dighiero, 1998). Avrameas and Ternynck

(1995) urged caution in that this is an assumption that needs to be experimentally verified. Some evidence can be found in the literature to support the concept of the monospecific immune antibody. Upon intentional immunisation and boosting of a mouse with TNP-KLH it was found that the resulting repertoire of IgG-secreting cells reactive with this antigen was highly specific, showing only 0.1-0.4% cross-reactivity with a variety of antigens in a chamber ELIspot assay (Klinman, 1994).

In summary, it is evident that using a variety of methodologies, antibodies have been observed to be polyreactive (observed to react with more than one antigen). The perception that an antibody is polyreactive, nonetheless, is very much dependent upon the method employed for its analysis. It depends on the sensitivity of the assay and also the number of antigens employed. It also depends on how the antibody was treated (such as exposure to low pH during purification). As a result, there is no clear consensus as to the meaning, relevance or usefulness of these observations.

With regard to their relevance, one extreme view is, that all observations of polyreactivity are artifactual, and due to the means of analysis employed (Cohn, 1992). These observations are then assumed to have no bearing on the physiology of antibodies. Other authors, taking the observations at face value, have speculated as to their implications for the physiological role(s) of antibodies (see section 1.2.6).

The usefulness, in general, of a classification system, whereby Mabs are polyreactive if shown to react with more than one antigen but otherwise are said to be monoreactive, can be questioned. It has not lead to any agreement as to the relative sizes of these two repertoires. Casali's group has provided evidence that B1a and B1b cells produce polyreactive antibody (see section 1.2.4). As these are minor B cell subsets, it may be concluded that, the majority of B cells produce monoreactive antibody. At the other extreme, Avrameas and Ternynck (1995) have stated that all preimmune B cells are polyreactive to some extent. As a second example, comparison of the structures of antibodies classified as polyreactive, with those classified as monoreactive, have not resulted, so far, in any consensus as to the structural differences between these two groupings (section 1.2.5).

The usefulness of such a classification system might be improved by taking probability theory into account. To illustrate this, imagine a hypothetical panel of 10 Mabs, of equal polyreactivity, tested against a limited and randomly chosen panel of 10 antigens. On the law of averages one would expect to get a distribution of reactivities (see Fig. 1.5). The crude classification system mentioned above would, as a result, incorrectly classify 3 of this panel of Mabs as monoreactive. Therefore, any useful classification of Mabs as monoreactive or polyreactive must involve a statistical analysis. Such statistical analyses are rarely if ever carried out. Also, for a sensitive statistical test, capable of efficiently discriminating between polyreactive and monoreactive antibodies, one would need to use a very large panel of antigens. The immunoblots of organ extracts already referred to may fulfil this criterion.

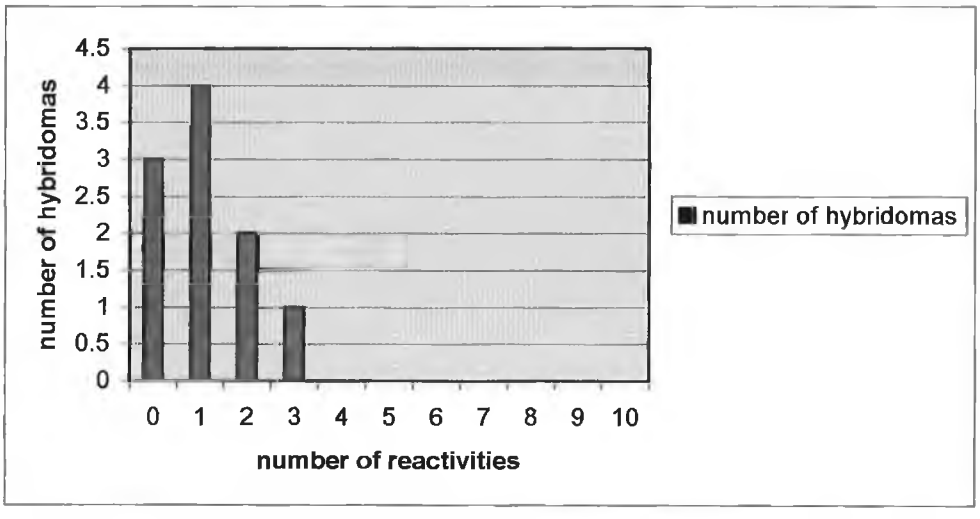


Fig. 1.5: *A hypothetical situation is presented. A panel of 10 Mabs of equal polyreactivity (the probability of any Mab reacting with any antigen (p) = 0.1. The probability of any Mab not reacting with any antigen (q) is 0.9) are each tested for reactivity with a panel of 10 antigens. The number of Mabs expected to react with 0, 1, 2, 3 .... 10 of the 10 antigens are depicted in the above graph. These values were calculated using the following binomial model:* $P_n = {}^{10}C_n \times p^n \times q^{10-n}$. *P is the probability of a Mab reacting with n of the antigens.*

The classification of Mabs as either polyreactive or monoreactive also gives the impression that polyreactivity is qualitatively absolute and disregards the possibility that B cells differ quantitatively in their degree of polyreactivity. Evidence can be found that the degree of polyreactivity varies widely among preimmune B cells. Grandien *et al.* (1994) analysed a randomly chosen panel of 128 hybridomas generated from B cells from bone marrow. These were analysed by ELISA for reactivity against a panel of eight antigens. From a potential total of 1024 positives 142 positives were obtained. A statistical analysis of these results is presented in Fig. 1.6

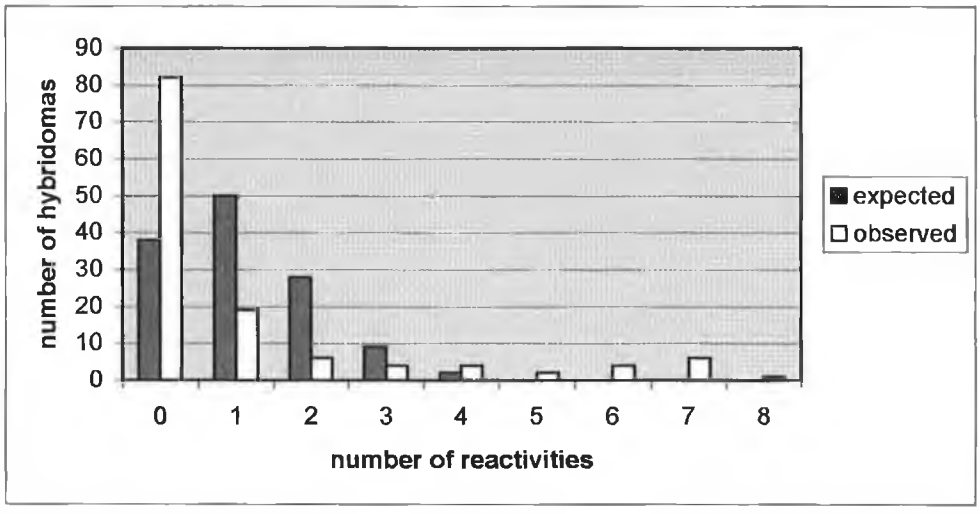


Fig. 1.6: *The null hypothesis to be tested is that the 128 Mabs analysed by Grandien et al. (1994) are equally polyreactive. Under this hypothesis the probability of a Mab reacting with an antigen (p) is 0.138 (142/1028). The probability of a Mab not reacting with an antigen (q) is thus 0.862. The expected distribution of Mabs, plotted above, is then calculated based on the binomial distribution* $P_n = {}^8C_n \times p^n \times q^{8-n}$. *The null hypothesis is then tested, by comparing the observed and expected distributions, using a '*χ^2 *goodness-of-fit test' (Ostle and Malone, 1988). When this test is carried out the null hypothesis is rejected at the 95% confidence level. It is accepted that the Mabs differ in their degree of polyreactivity. The observed distribution does not indicate two discrete populations. This suggests that rather than two discrete populations of Mabs (one polyreactive and one monoreactive), one is observing a continuous gradation of polyreactivity.*

1.2.3 Characteristics of polyreactive antibodies: There is broad agreement with regard to a number of characteristics of antibodies classified as polyreactive. They tend to be produced by preimmune B cells. In general, they tend to react with antigen, with only modest affinities of between $1x10^{-6}$ – $1x10^{-8}$M. They tend to utilise gene segments in germline configuration (Dighiero, 1997). Most importantly, perhaps, polyreactive antibodies are not simply "sticky" but at the monoclonal level show very fine discrete sets of specificities. This has been demonstrated, most elegantly, using a newly developed immunoblot assay in which monoclonal polyreactive antibodies are assayed for reactivity against many 100's of antigens at once (Nobrega *et al.*, 1993; Haury *et al.*, 1994). There is some disagreement, however, with regard to the prevalent isotype among polyreactive antibodies. Casali and Schettino (1996) have indicated that the IgM isotype dominates at least among the natural antibody repertoire. While this may simply reflect the dominance of the IgM isotype, in general, the results of Klinman (1994) tend to refute this explanation. He found using a chamber ELIspot assay that the IgG repertoire of naturally activated splenic B cells (in mice) is much less cross-reactive than the IgM repertoire. In contrast to the view of Casali and Schettino (1996), Avrameas and Ternynck (1995) have indicated that, for the natural antibody repertoire of mice and men, IgG and not IgM, is the predominant isotype of polyreactive antibody. Nevertheless they have indicated that, at least in mice, this IgG activity can be masked in normal serum and is only manifest upon purification of the IgG from the serum.

1.2.4 Cell surface markers for polyreactivity: Evidence is available that polyreactive B cells segregate with the CD5+ B cell subset (Nakamura *et al.*, 1988a; Casali and Notkins, 1989; Bhat *et al.*, 1997). Nevertheless, this is a controversial issue and more recent evidence appears to indicate that this cell surface marker may not be indicative of polyreactivity. For example, Chen *et al.* (1995) found that both CD5+ and CD5- B cells from the peripheral blood of healthy humans could produce polyreactive antibody although the CD5+ subset did contain a greater frequency of cells secreting polyreactive antibody.

A possible explanation for such contradictory results may lie in recent advances in our understanding of B cell subsets. B cells have been divided into two broad subsets based on the expression or lack of expression of the CD5

antigen (reviewed by: Herzenberg *et al.*, 1986; Hiyakawa and Hardy, 1988; Kipps, 1989; Kantor, 1991; Lydyard *et al.*, 1993). These cells are not only phenotypically distinct but they respond differently to cytokines and have different bodily distributions. As previously suggested, they may express different repertoires of antibodies and hence may fulfil different physiological roles (Lydyard *et al.*, 1993). There also is evidence that these subsets have completely different cell lineages although some workers have suggested that the CD5 antigen is simply an activation marker on B cells (Lydyard *et al.*, 1993). More recently it was found (mice and humans) that a third B cell subset exists, which appears to be identical to the CD5+ subset in every way except for its lack of expression of the CD5 antigen (Kasaian and Casali, 1993). In modern parlance this subset is referred to as the B1b subset, the CD5+ B cell subset is the B1a subset with the remaining B cells being referred to as the B2 subset. As a result it is possible that, while polyreactive B cells do not segregate with the CD5+ B cell subset, they do segregate with cells of the B1 lineages (Kasaian and Casali, 1993). This viewpoint is, however, made less tenable by the finding (Grandien *et al.*, 1994), that emergent B cells in the bone marrow are very polyreactive. All available evidence points to the bone marrow being a source of B2 cells only (Kantor, 1991).

A better phenotypic marker for polyreactive B cells may be their ability to bind antigen. Chen *et al.* (1995) separated enriched B cells based on their ability to bind β-galactosidase, a randomly chosen antigen. 30% of B cells were positively selected and the frequency of B cells producing polyreactive antibody was 10 times greater in this population than the remaining B cells.

1.2.5 Structural correlates for polyreactivity: Efforts have been made to compare antibodies classified as polyreactive or monoreactive in order to determine if any discrete structural features predispose to one characteristic over the other. One suggestion is that the IgM isotype is an important structural correlate for polyreactivity (Ghosh and Campbell, 1986). However, although polyreactive antibodies may frequently be of the IgM isotype the relationship is unlikely to be a causal one. The elegent gene shuffling experiments of Ichiyoshi and Casali (1994) demonstrated that the variable regions of a polyreactive IgM antibody

could be successfully grafted on to an IgG constant region with retention of polyreactivity. The relationship between polyreactivity and IgM isotype (if any) is more likely a reflection of the fact that the IgM isotype is associated with the preimmune repertoire and IgG with the immune repertoire.

There are, presumably, some features of an antibody binding site that predispose to polyreactivity. Polyreactive Mabs tend to utilise minimally mutated (germline) V_H gene segments. This cannot be sufficient for polyreactivity as monospecific B cells also utilise germline genes and IgG and IgA polyreactive antibodies contain somatic mutations (Harindranath *et al.*, 1993; Ichiyoshi and Casali, 1994). It was postulated that the gene segments coding for polyreactive antibodies are distinct from those encoding specific antibodies, but there is no conclusive evidence to suggest that V_H gene segments preferentially segregate with polyreactive or monospecific B cells (Ichiyoshi and Casali, 1994; Bhat *et al.*, 1997).

There is some evidence to suggest that the CDR3 of the heavy chain may be an important structural correlate for polyreactivity. Kipps *et al.* (1993) have shown that CLL cells express a very skewed repertoire of D_H and J_H gene segments relative to a normal B cell repertoire. Using a recombinant approach they demonstrated that replacing the CDR3 of a polyreactive CLL antibody with a variety of randomly chosen CDR3s resulted in the loss of polyreactivity. They have also shown that the CDR3 of CLL antibodies tend to be significantly longer than those in a general B cell repertoire and have suggested that this is a structural correlate for polyreactivity (Kipps *et al.*, 1993). The chain shuffling experiments of Ichiyoshi and Casali (1994) also suggest that the CDR3 can play an important role in polyreactivity. They demonstrated that replacing the CDR3 of a monospecific antibody with the CDR3 of a polyreactive antibody was sufficient to generate a chimaeric antibody that had lost its monospecificity. Although the CDR3 of this particular polyreactive antibody was very long they cautioned that in general the CDR3s of polyreactive antibodies appear to be diverse in terms of length and amino acid composition. Hence, they suggested that length may not be the sole characteristic of the CDR3 responsible for polyreactivity or that different structures outside of the CDR3 can be responsible for polyreactivity in different antibodies (Ichiyoshi and Casali, 1994). The value of these observations on the importance of CDR3 for polyreactivity may

be questioned given that it appears to be important for antibody binding in general (see section 1.1.1.3).

As suggested in section 1.2.1, general characteristics of the antibody binding site such as its flexibility ('plasticity') may be responsible for polyreactivity. Padlan (1994) has suggested that the antibody binding site may simply be very plastic in polyreactive antibodies. It has also been suggested that very large binding sites may be a characteristic of polyreactive binding sites. In this regard analysis of the overall three-dimensional structure of polyreactive antibodies may be useful. Ramsland *et al.* (1997) have made a start in this direction by homology modelling the three-dimensional structures of three polyreactive binding sites. No common structural feature was found that might distinguish them from monospecific antibodies. Labrousse *et al.* (1997) have approached the problem by analysing the effect of physicochemical parameters, such as temperature and pH, on the binding of monoreactive and polyreactive antibodies. They found that polyreactive binding was much more sensitive to variations in these parameters and concluded that polyreactive binding sites are more plastic than monoreactive.

It was suggested that polyreactivity might be dependent upon post-translational modification of the antibody molecule (Fernandez *et al.*, 1997 and 1997a). These authors have shown that polyreactive binding is dependent on glycosylation of the antibody molecule at least in some instances.

Finally, it has also been suggested that it is not the structure of their binding sites, but rather the nature of the epitopes that they recognise that distinguishes polyreactive antibodies from their monospecific counterparts. Polyreactive antibodies may recognise 'public epitopes' carried on many antigens. In this regard, Tchernychev *et al.* (1997) have provided evidence that the epitopes for natural polyreactive antibodies are rich in proline. They have suggested that structures containing proline can serve as conformation dependent 'public epitopes' for polyreactive antibodies.

1.2.6 Physiological roles of polyreactive antibodies: The concept of polyreactivity has been incorporated into conventional thinking (clonal selection) on the role of antibodies in acquired immunity. For example, it has been argued that a preimmune polyreactive repertoire would provide better coverage of the

potential universe of antigens and would provide a template upon which somatic hypermutation would act to increase specificity and affinity. Several authors have discussed this concept (Harindranath *et al.*, 1993; Dighiero, 1997 and 1997a).

As previously discussed, antibody monospecificity is a basic tenet of clonal deletion theories. Therefore, it is curious that some authors have considered the notion that polyreactive B cells may play a role in deletion. Chen *et al.* (1995 and 1996) have suggested that polyreactive B cells with many anti-self reactivities could act as antigen presenting cells for T cells with deletion of the T cell or induction of anergy.

Other researchers have postulated that polyreactive antibodies may fulfil defensive roles distinct from those of monoreactive antibodies. Since, the natural antibody of the serum (and bodily secretions) is reactive against many foreign pathogens (presumably due to its polyreactive antibody component), the suggestion is that this antibody forms an antibody-mediated innate defensive mechanism against bacteria and other pathogens (Harindranath *et al.*, 1993; Casali and Schettino, 1996; Quan *et al.*, 1997). These authors hypothesize that B2 cells are the B cells committed to conventional immune responses, while B1a and B1b cells function in the antibody-mediated innate defensive mechanism. In support of this concept the theory advanced is that the B1a cell is a rather primitive B cell lineage as it is the first to appear in phylogeny and that it may represent a stepping stone between innate immunity and acquired immunity (Lydyard *et al.*, 1993). Bouvet and Dighiero (1998) have also suggested the concept of a layered humoral immune system. They posit an initial, primordial, polyreactive, autoreactive, antibody molecule, which may have been of use in the clearance of degraded auto-antigen. Gene duplication and mutation followed by auto-antigen driven natural selection resulted in a simple preliminary immune system which functioned in cell debris clearance. Due to its polyreactivity it developed a secondary protective role by binding to pathogens. Later in evolution modern monospecific antibodies evolved to fight off pathogens which had mutated to avoid binding the natural antibodies. Modern vertebrates have maintained the primordial immune system as the modern immune response to pathogen has to be induced and to prevent pathologic autoimmunity (by binding to and masking autoantigen).

A number of authors have gone so far as to speculate that polyreactive antibodies may fulfil roles of a non-defensive nature. It was noted in section 1.2.1 that polyreactive antibodies are essential to the setting up and functioning of the CIS. For example, the V_H genes expressed first during ontogeny are highly polyreactive in so far as they show very high idiotypic connectivity with each other (Kearney and Vakil, 1986; Varelo and Coutinho, 1991). This developmentally regulated expression of antibody gene segments may facilitate the early organisation of an idiotype network (Varelo and Coutinho, 1991). This network is then maintained (in part) by recruiting from the emergent B cell population, those B cells showing polyreactivity (Varelo and Coutinho, 1991). (This recruitment into the CIS, of polyreactive antibodies, may actually be the reason that the PIS and hence immune antibodies, tend to show monoreactivity). From the point of view of host defense the CIS may serve no other role than the prevention of pathologic autoimmunity (Coutinho and Avrameas, 1992). However, the suggestion was also made that rather than playing a role in host defense the CIS may in fact play a role in the homeostasis of all other biological systems (Coutinho and Avrameas, 1992). Stewart (1992) has proposed that immunoglobulins arose in evolution for this purpose and that their defensive role was a relatively late evolutionary development.

1.3 Monoclonal antibody production

As mentioned at the start of this chapter, antibody binding sites are considered the paradigm for specific biomolecular interactions. **The biotechnological importance of antibodies and antibody derivatives is predicated upon this perception.** In basic, applied and clinical research, they are important components of many analytical tools. Their use in ELISAs allows the detection and quantification of ligands in complex biological matrices. HIV diagnosis is frequently dependent upon detection of the p24 antigen by ELISA. The detection of pesticides in foodstuff extracts, currently assayed in the laboratory by relatively cumbersome tools such as high pressure liquid chromatography (HPLC), will soon be assayed, in the field, using ELISA-based 'dipsticks'. The use of monoclonal antibodies allows the visualization of ligands in biological matrices (e.g. immunocytochemistry). This is the case, frequently, in post-mortem analysis of tissue samples, for viral infections.

Antibodies are also expected to find many therapeutic uses in the future. 30% of all biological molecules currently undergoing clinical trials are recombinant antibodies (Hudson, 1998). They may find a role in cancer therapy, targeting toxins to tumours (immunotoxins and genetic immunotoxins) (Chen and Marasco, 1997). Other, more speculative proposals, include their use to target gene vectors to specific tissues or cell types (Roth and Cristiano, 1997), which is a prerequisite for many forms of gene therapy.

However, in light of the observations of polyreactive antibodies reported in section 1.2, the perception that antibodies are invariably specific is no longer tenable. While one may argue about the relevance of such observations to the physiological roles of antibodies (e.g. Cohn, 1992), from a biotechnological point of view it is the observation in and of itself that is important. For example, if one observes that **an antibody binds in solution to more than one ligand**, one clearly could not use the antibody in a competitive ELISA to **detect and quantitate a specific ligand**. It is with this point of view that, in this section, monoclonal antibody production by immortalisation of B cells or repertoire cloning is reviewed. It is suggested that the means of production of Mabs greatly influences the probability of generating a specific Mab.

1.3.1 B cell immortalisation: The conventional approach for the production of monoclonal antibodies relies on the immortalisation and subsequent cloning of B cells, prior to screening secreted antibody, for the reactivity desired. A number of approaches for immortalisation are possible. Murine B cells are normally immortalised by fusing B cells with myeloma (a cancerous cell line of lymphoid origin) cells. This results in a population of cells referred to as hybridomas with the twin attributes of immortality (from the myeloma) and antibody secretion (from the B cell partner). The population of hybridomas can be cloned, by culturing under limiting dilution conditions, and then screened

Human B cells, normally, are immortalised by fusion with either a myeloma or preferably a heteromyeloma (Kozbor *et al.*, 1986; Zanella *et al.*, 1992). They may also be immortalised using Epstein Barr Virus (EBV) (Steinitz *et al.*, 1977).

1.3.1.1 Fusion: Two methods are now predominantly used to induce membrane fusion. These are electrofusion and the chemical fusogen polyethylene glycol (PEG).

Kohler and Milstein (1976) were the first to use PEG for cell fusion. PEG causes two obvious effects on cells: i) dehydration and shrinkage of cells and ii) cellular aggregation (Lucy, 1978; Klebe and Bentley, 1987; Hui and Stenger, 1992). Dehydration of cells is due to hydration of the hydrophilic PEG molecules. Fusion may occur, during PEG dilution (cell rehydration), due to weakening of cytoskeletal and membrane protein restraints and subsequent increase in membrane fluidity (Lucy, 1978). The driving force for coalescence of the adjacent bi-layers is not obvious but PEG may introduce structural deformities into the bi-layer (Hui and Stenger, 1992).

Electrofusion was first applied to lymphocytes by Bishoff *et al.* (1982) and the first monoclonal antibodies produced by Karsten *et al.* (1985). Two processes are involved in the electrofusion of freely suspended cells: i) formation of cell chains (pearl chains) to induce membrane contact and ii) application of an electrical field pulse of sufficient strength to cause reversible membrane breakdown (Zimmerman, 1982). A number of methods have been used to induce cell alignment, dielectrophoresis being the preferred method (Zimmermann, 1987). The following provide excellent indept reviews of the theory of dielectrophoresis and electrical membrane breakdown: (Zimmermann,

1982; Zimmermenn, 1987; Zimmermann *et al.*, 1990; Perkins *et al.*, 1989; Neil and Zimmermann, 1993).

Both these techniques are quite inefficient with PEG immortalising only one out of every 100,000 B cells and electrofusion one out of every 10,000. It should also be noted that in both cases, the resulting population of hybridomas is not a random sample, of the B cell population, as the fusibility of cells varies with their state of differentiation. The optimum state of differentiation is unknown but it is clear that activated cells are preferentially fused over resting cells (Shay, 1985).

*1.3.1.2 **EBV transformation***: EBV is a human lymphotrophic herpes virus that can activate and transform human B cells (Crawford, 1985). Although all resting B cells appear to have the receptor for EBV infection, there are conflicting views on its expression during the cell cycle. Nonetheless, transformed B cells appear to be a random sample of the resting B cell population (Casali and Notkins, 1989).

Transformation of B cells with EBV is a well-documented procedure and details can be found in Crawford (1985). Transformation efficiencies of up to 40% of all input B cells have been reported (Chan *et al.*, 1986) although 1% appears to be a reasonable average.

1.3.2 **B cell populations for immortalisation:** In the case of murine B cells, generating the required B cell population prior to fusion does not present any problems, in general. One simply hyperimmunises a mouse with the ligand of interest, sacrifice it three days after the final boost, and fuse its splenocytes with a myeloma cell line. Having undergone an immune response, the splenocyte population will be enriched in B cells secreting antibody of the desired reactivity, which, being activated, will be fused preferentially over the resting preimmune B cells. **The activated immune B cells can reasonably be expected to be of high affinity and specificity**. Although this is the preferred means of generating a B cell population prior to fusion, in certain circumstances a technique referred to as *in vitro* immunisation (IVI) is said to be preferable. IVI is discussed in section 1.3.2.2.

Unfortunately, generating a suitable source of human B cells prior to fusion is not as trivial a task as in the case of the mouse. The major problem is an ethical one; one simply cannot inject most ligands into a human volunteer to generate an immune response as in the case described above for the mouse. Thus, in general, one must resort to immortalising preimmune B cells obtained from peripheral blood or immunise peripheral blood lymphocytes *in vitro.*

1.3.2.1 ***Preimmune B cells***: Preimmune B cells are not immortalised well by fusion (see section 1.3.1.1), so EBV is generally used. Steinitz *et al.* (1977) advised antigenic pre-selection of lymphocytes, of the desired reactivity, from the preimmune population, to remove problems of clonal competition from irrelevant cell lines. This appears to be the preferred strategy with antigenic post-selection also being used (Bron *et al.*, 1984).

Other strategies using preimmune B cells include use of the EL-4 culture system (see Zubler *et al.*, 1985; Zubler *et al.*, 1987; Wen *et al.*, 1987) to activate pre-selected B cells prior to immortalisation by electrofusion (Steenbakkers *et al.*, 1994). The CD40 culture system (see Banchereau *et al.*, 1991) has also been used in combination with EBV transformation to produce monoclonal antibodies (Peyron *et al.*, 1994). The main reason for activating B cells in these culture systems appears to be that they facilitate class switch of preimmune IgM antibodies to IgG or IgA.

One potential problem, with strategies involving the use of preimmune B cells, is that they may suffer from polyreactivity. The results of Chen *et al.* (1995) also suggest that antigen-selection of B cells will enrich for polyreactive B cells.

1.3.2.2 ***In vitro immunisation***: Moller and Borrebaeck (1988) have defined this type of culture system as a primary immunisation system i.e. one that allows an antigen-induced, antigen-specific response. The area has been extensively reviewed in the past (see Reading, 1982; Borrebaeck, 1986; James and Bell, 1987; Borrebaeck 1988 and 1988a; Borrebaeck, 1993) and will only be briefly introduced here. Instead the main thrust of this section will be to point out areas of concern with regard to the suitability of this technique for monoclonal antibody production.

Mishell and Dutton (1966) first achieved antigen-specific activation of B cells. Since that time, a broad range of protocols leading to the antigen-specific activation of B cells have been published. Although differing in the fine-detail, most published protocols consist of three main components. Firstly, a source of immunocytes is required. For murine systems unfractionated splenocytes are almost invariably used. For human systems peripheral blood lymphocytes are generally used and for an optimal response need to be pretreated with l-leucyl-leucine methyl ester. This removes the lysosomal-rich cell fraction (Borrebaeck *et al.*, 1988). Secondly, a source of lymphokines is required to facilitate the immune response. In general conditioned media is used for this purpose. Conditioned media which have been applied in murine systems include thymocyte-conditioned media (Luben and Moller, 1980), mixed lymphocyte-conditioned media (Borrebaeck, 1983), supernatant of an EL4 thymoma cell line (Ma *et al.*, 1984) and splenocyte-conditioned media (Reason *et al.*, 1987). In human systems a number of different polyclonal activators (poke weed mitogen (PWM), phytohaemagglutinin (PHA), concanavalin A (con A), lipopolysaccharide (LPS)) have been used to stimulate lymphoid cells and the resulting conditioned media is collected and utilised. The third component required, is the antigen against which an immune response is desired.

In vitro immunisation was first used to provide activated B lymphocytes prior to fusion, by Hengartner *et al.* (1978) and Luben and Moller (1980). Borrebaecks group, in particular, have advocated its application for the antigen-specific activation of human B cells prior to fusion. This is because ethical considerations preclude *in vivo* immunisation. Other characteristics of *in vitro* immunisation, which supposedly make it an attractive proposition for immunising human or indeed murine B cells, include the following. Firstly, the culture period prior to fusion, generally, does not exceed five days. Secondly, only very small amounts of antigen are required. Concentrations used have varied from 1ng/ml to 100μg/ml (Borrebaeck, 1986). Thirdly, immune responses to self-antigens can be obtained and immunogenic epitopes, which dominate *in vivo*, are moderated. Finally, toxic antigens can be used *in vitro* but not *in vivo*. **Regardless of the above, the suitability of this technique for monoclonal antibody production can be questioned.**

Firstly, it is by no means certain that *in vitro* immunisation is indeed an accurate mimic of the *in vivo* immune response. While, it cannot be denied that *in vitro* immunisation protocols give rise to an increase in frequency of antigen-reactive B cells, many of its other characteristics are foreign to *in vivo* responses. i) *in vitro* immunisations occurring in suspension obviously cannot recreate the organised structure of an immune organ, this organisation being assumed to play a role in the *in vivo* immune response (Zubler *et al.*, 1987). ii) 2-mercaptoethanol, which is toxic *in vivo*, is an essential nutrient in all published *in vitro* immunisation protocols. iii) In certain *in vitro* immunisation protocols (e.g. Borrebaeck and Moller, 1986) T cells, which are essential *in vivo*, have been demonstrated to be superfluous. A related observation is that hapten-induced, hapten specific immune responses appear to occur *in vitro* (Stahl *et al.*, 1995). iv) Antigen-induced, antigen-reactive immune responses, at least in certain protocols, are superimposed on a very large polyclonal B cell response (Borrebaeck and Moller, 1986). v) Most crucially, however, it should be pointed out, that clonal expansions of self-reactive B cells, one of the cited advantages of *in vitro* immunisation, is totally at odds with one of the most distinctive characteristics of the immune system *in vivo*. In healthy mammals clonal expansions of self-reactive B cells does not occur! This characteristic may indicate that naturally activated B cells including/and/or B-1 cells may be preferentially activated *in vitro*.

Secondly, the robustness and reproducibility of the technique has been queried. A number of authors such as Uthoff and Boldicke (1993) and Brams *et al.* (1993a) have failed to generate antigen-reactive immune responses using published *in vitro* immunisation protocols. As noted by Grandien *et al.* (1994) referring to B cell culture systems "Widely discrepant results have been observed and reported after *in vitro* exposure of B cells to Ig ligands: death of the cells, complete activation to increassed DNA synthesis (which some interpret as repair), activation of responsiveness to various lymphokines, etc., let alone all the biochemical phenomenology on "signalling pathways" and early activation events. Differences in normal cell populations or transformed cell lines, in the ligands (antibodies) used, in the culture conditions, in the assays employed, all can be invoked to explain such discerpancies, but do not entirely remove the widespread conviction that *any* result can be obtained, with the

consequent reservations as to the value of the observations for B cell physiology *in vivo*."

Thirdly, the common assertion that the five-day culture period *in vitro* is a tremendous advantage over the 30-day culture period required for an immune response *in vivo* is blatant misrepresentation. *In vitro* immune responses are primary responses, in general, and the 30-day period referred to, allows a secondary immune response to occur *in vivo*. In fact, a primary immune response will occur *in vivo* in three to five days.

Fourthly, and perhaps of greatest practical consequence, since *in vitro* immunisation protocols generally mimic primary immune responses only, the resulting antibodies might be expected to be of low affinity and perhaps also of broad specificity. At worst, if for example B-1 cells are preferentially activated, the resulting monoclonal antibodies can clearly be expected to be polyreactive. Unfortunately there is a lack of information with regard to these characteristics in the published literature although Moller *et al.* (1990) have reported generating an IgM antibody of high affinity ($3x10^9$ M^{-1}) using an *in vitro* immunisation protocol. It should also be noted that there have been two reports in the literature of secondary immune responses being generated *in vitro* from preimmune human B cells as defined by class switch and possibly affinity maturation (Brams *et al.*, 1993 and 1993a; Zafiropoulos, *et al.*, 1997). Nonetheless, until the monoclonal antibodies produced by *in vitro* immunisation are more fully characterised, with regard to specificity and affinity, doubts about its general utility for monoclonal antibody production may be justified.

1.3.3 Immunological repertoire cloning: As pointed out in section 1.1.2, the advent of PCR has facilitated the cloning of gene segments, encoding V_H and V_L domains from hybridomas. This technology has been brought to its logical conclusion by utilising it to clone repertoires of rearranged gene segments encoding V_H and V_L domains from B cell populations (Fig. 1.7). These amplified gene segments are, in general, randomly recombined in ScFv format by e.g. slice-overlap-extension (Krebber *et al.*, 1997) to form combinatorial libraries of genes, encoding hopefully a diverse repertoire of binding site reactivities. The resulting repertoire can be cloned into a vector and maintained

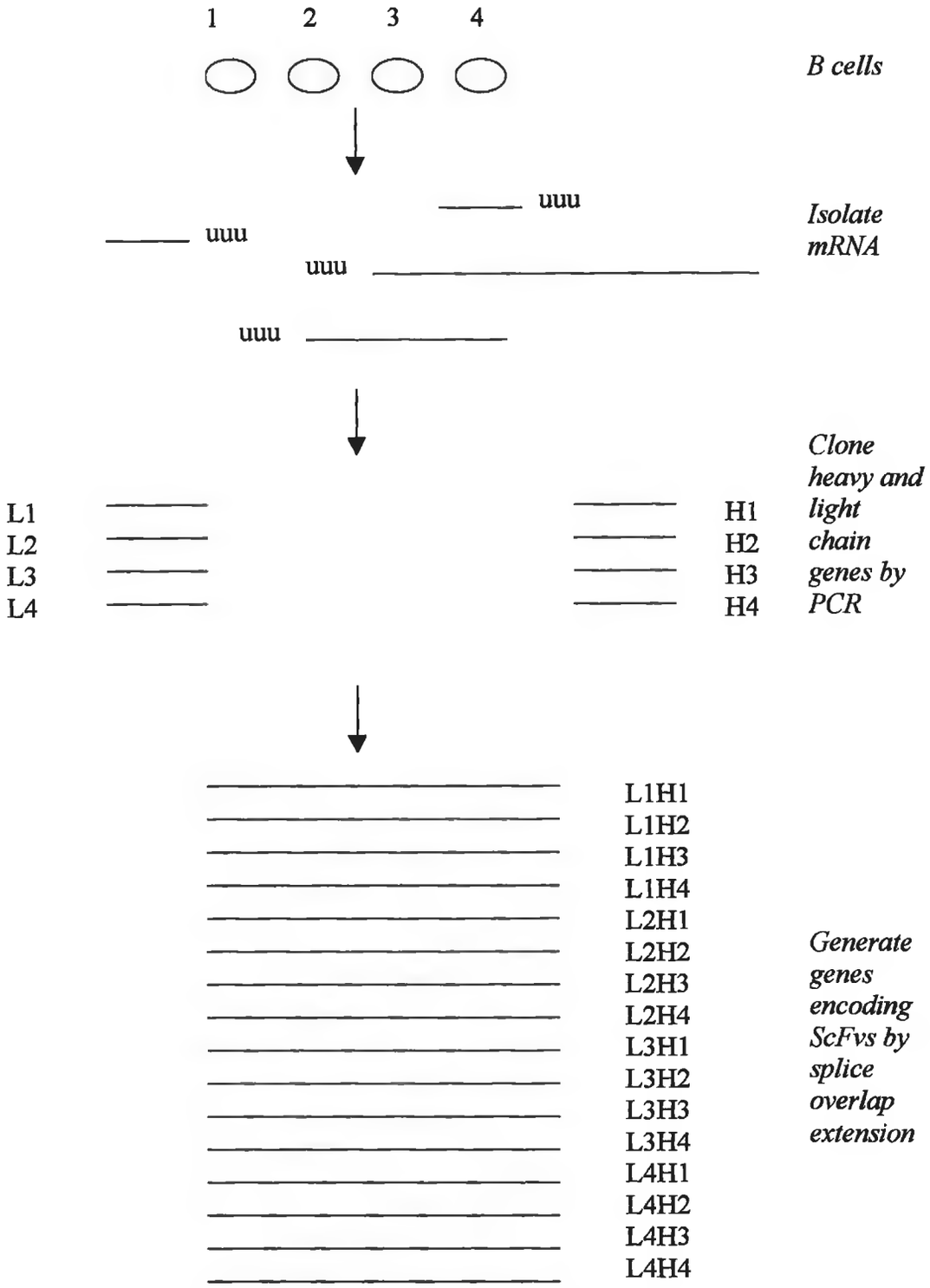


Fig. 1.7: *Steps in the generation of a combinatorial antibody library from 4 B cells. Note that the complexity of the library varies to a first approximation with the square of the number of B cells used to create the library.*

indefinitely in *E. coli*. This overall process is referred to as "immunological repertoire cloning" (Persson *et al.*, 1991). These repertoires can be probed, by screening or selection, to isolate genes encoding a desired reactivity. Isolated genes, subsequently, can be expressed in *E. coli* for monoclonal antibody production. 'Proof-of-principle' experiments have clearly demonstrated the feasibility of the approach. Ward *et al.* (1989) isolated V_H domains, reacting with lysozyme or keyhole limpet haemocyanin, with affinities of around $2x10^{-8}$ M from secondary immune repertoires (see section 1.3.3.1). Huse *et al.* (1989) isolated Fabs reacting with p-nitrophenyl phosphonamidate, from a secondary combinatorial library, with apparent affinities of 10^{-7} to 10^{-9}M. Nissim *et al.* (1994) have isolated ScFvs reacting with various foreign and self-antigens from a naïve human immune repertoire (see section 1.3.3.1). The technology has been extensively reviewed recently (Hoogenboom, 1997; Aujame *et al.*, 1997; Griffith and Duncan, 1998; Hudson, 1998) and the reader is referred to these for a more in-dept treatment of the technology, which is only briefly outlined below. Subsequent sections address the following questions. What B cell populations should we use for cloning? How can we best probe the resulting repertoires for the desired reactivity? What are the advantages of this system over B cell immortalisation?

1.3.3.1 B cell populations: The first choice to be made is between a B cell population from an individual immunised with the antigen of interest or from an unimmunised (naïve) individual. A B cell population from an immunised individual will be enriched in somatically mutated V_H -V_L combinations selected for high affinity with the desired antigen. The resulting secondary repertoire can reasonably be expected to be enriched in high-affinity binders, relative to a repertoire cloned from a naïve individual (naïve repertoire), even with the random recombination inherent in repertoire cloning, which scrambles up the *in vivo* pairing of heavy and light chains (see Gherardi and Milstein, 1992). The disadvantage of this process of course is that separate secondary libraries must be generated for each antigen of interest. A naïve library can be used again and again for many different antigens but can be expected to contain reactivities of only moderate to weak affinity, the mean affinity increasing with size of the naïve repertoire. On a positive note, it is possible to treat genes isolated from a

naïve library (or indeed any other kind), which are of insufficient affinity, as a "lead" gene. Strategies to randomly mutate such "leads", generate libraries from which genes encoding higher affinity reactivities can be isolated. Such strategies include, random mutations using mutator strains of *E. coli*, targeted mutations, chain shuffling, error-prone PCR and sexual PCR (Bradbury, 1997).

The second choice to be made is from which organ to obtain B cells. The generation of secondary libraries, from immunised mice, requires that the B cells are isolated from a secondary organ and the spleen is invariably used. For generating naïve repertoires it appears to be accepted that bone marrow B cells are the theoretically best source of B cells. This appears to be based on the belief, that bone marrow B cells are generated, and proliferate, in an antigen-independent fashion, and hence represent an unbiased, highly diverse, source of rearranged immunoglobulin genes. In actual fact no stage of lymphopoiesis appears to be antigen-independent (Grandien *et al.*, 1994a). Furthermore, studies of characteristics, such as diversity, although in their infancy, have indicated that the splenic B cell repertoire, of mice, is as diverse as the bone marrow repertoire with the potential advantage that it appears to be less polyreactive (Grandien *et al.*, 1994a). For humans the only ethical choice is to obtain B cells from peripheral blood.

It should be mentioned that completely synthetic libraries, generated independently of any B cell population, are considered by many authors to be the best strategy for repertoire cloning, as one has better control over the content of the library (Hudson, 1998).

1.3.3.2 ***Probing cloned repertoires***: Initially libraries were screened for reactivities of interest. Ward *et al.* (1989) generated V_H secondary libraries to lysozyme and keyhole limpet haemocyanin and cloned the library into an *E. coli* expression vector. Screening was carried out, by assaying the supernatants, from individual transformants, by ELISA. A similar strategy was used by Huse *et al.* (1989).

As pointed out by McCafferty *et al.* (1990) selection is a much more powerful means of probing libraries than screening. They demonstrated that by phage display of ScFvs, one ScFv, with a desired reactivity, could be selected from a million irrelevant ScFv, by affinity chromatography.

Phage display is based on the observation by Smith (1985) that peptides (and as subsequently shown proteins) could be fused to the coat protein of filamentous phage without completely abrogating their infectivity for *E. coli*. The strategy as applied to probing immune repertoires is depicted in Fig. 1.8 with specific reference to the Nissim library (Nissim *et al.*, 1994). pIII refers to a M13 (filamentous phage) coat protein. PelB is a bacterial leader sequence allowing export to the periplasm. C-myc refers to a short peptide tag derived from the C-terminus of the c-myc protein. This tag is recognised by the mouse monoclonal antibody 9E10. pHen1-Vλ3 is a phagemid vector, i.e. it contains the IG (intragenic) region from M13 thus allowing it to be packaged into a phage particle if the M13 structural proteins are provided in *trans*. An amber stop codon exists between *c-myc* and *gIII*.

The Nissim library was composed from 50 germline V_H gene segments, amplified in such a fashion as to introduce a random synthetic CDR3. The resulting product was cloned into the pHen1-Vλ3 vector generating a library encoding diverse PelB-ScFv-c-myc-pIII fusion proteins. The resulting library is propagated in *E. coli* TG1 and upon induction a specific ScFv-c-myc-pIII fusion protein is expressed in the periplasm of individual cells. If induction is accompanied by infection with a helper phage (a filamentous phage whose genome contains the genetic information for all its structural proteins but lacks an IG region so its genome cannot be packaged) a library of phage particles are excreted. Individual phage particles will contain both native pIII and a specific ScFv-c-myc-pIII on their surface and, as a genome, will contain the specific phagemid vector encoding the ScFv fused on their surface. Thus, affinity-purification of phage particles with a relevant antigen, (panning), enriches for phagemid vectors encoding ScFvs with antigen-binding activity. Affinity-purified phage can infect *E. coli* TG1 again to allow the propagation in *E. coli* of this enriched sublibrary. In general, this selection procedure is repeated twice. The final phage population is used to infect *E. coli* HB2151, which recognises the amber stop codon, and hence produces an ScFv-c-myc fusion protein, and the resulting *E. coli* clones are screened for the desired reactivity, by e.g. ELISA making use of the c-myc tag.

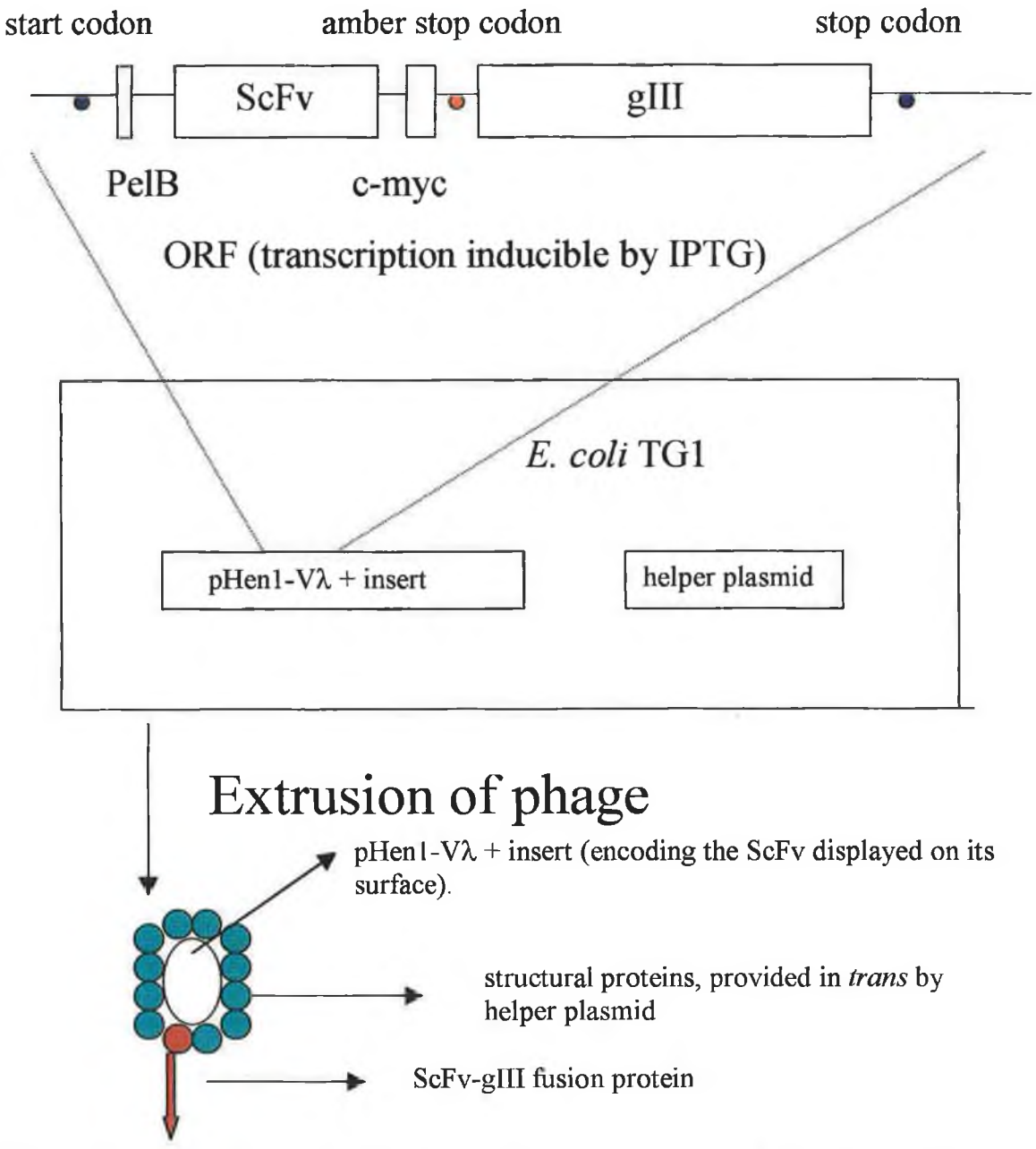


Fig. 1.8a: *One clone from an immune repertoire library has just been infected with helper phage and cultured in the presence of isopropyl β-D-thiogalactopyranoside (IPTG). This results in extrusion of specific phage particles, expressing on their surface, and encoding only one species of ScFv. Infection and induction of the entire library results in a mixture of phage particles, each expressing on its surface, and encoding, a different species of ScFv (phage library). Antigen can then be used to affinity-purify, from the phage library, those phage expressing and encoding ScFv with anti-antigen binding activity (see Fig. 1.8b).*

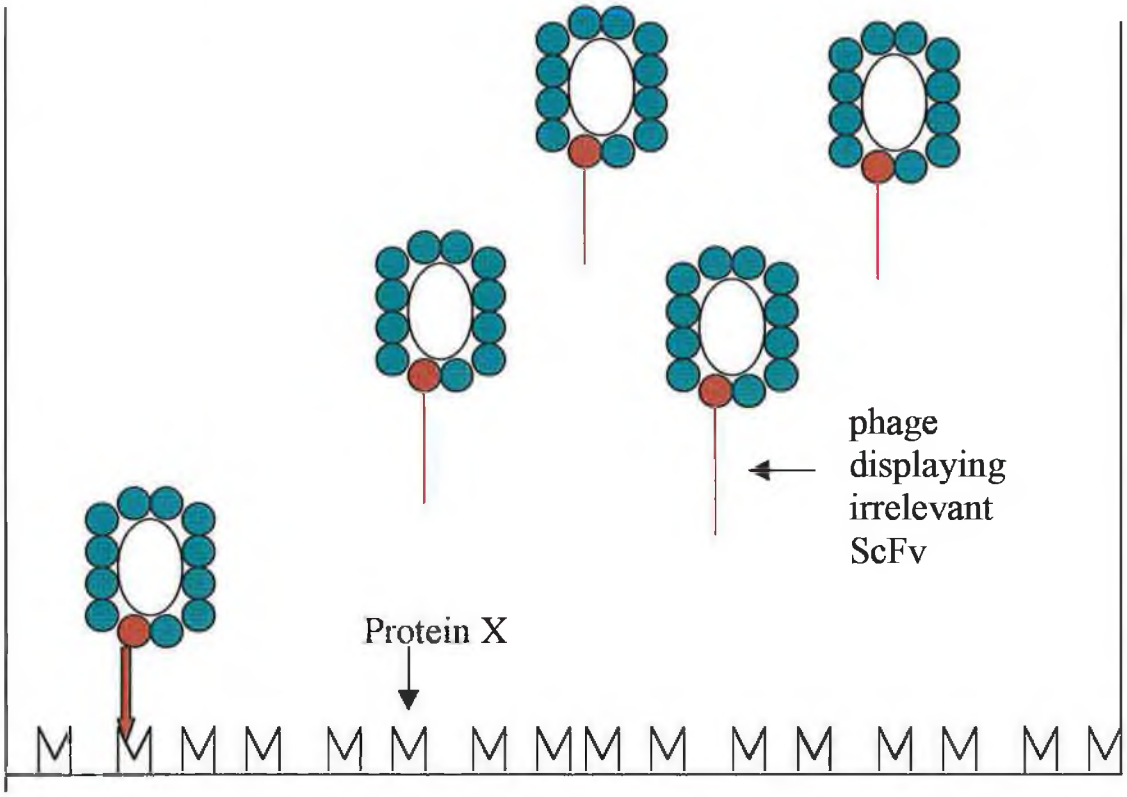


Fig. 1.8b: *Affinity-purification, of phage expressing and encoding ScFv with anti-protein X binding activity, from a phage library. The phage library is harvested from a culture of E. coli TG1, and are incubated on a plastic surface, coated with protein X. Phage expressing and encoding ScFv, reactive with protein X, are isolated by washing off irrelevant phage and then eluting bound phage. This population of phage (sublibrary) can be expanded, in E. coli TG1, prior to repeating the affinity-purification. Alternatively E. coli HB2151 can be infected to produce soluble ScFv-c-myc proteins. See text for additional details.*

*1.3.3.3 **Advantages of immunological repertoire cloning:*** A number of advantages are attendant upon this technology. From an ethical point of view animal suffering is reduced as naïve libraries have to be created just once and no immunisation is required. Equally, there are many pragmatic reasons for employing this technology. i) B cell immortalisation is a very inefficient process and hence does not provide comprehensive or easy access to the *in vivo* antibody repertoire. *In vitro* repertoires in *E. coli* are, however, easily and efficiently accessed by selection and screening. ii) As already indicated the generation of human monoclonal antibodies using fusion technology has had limited success and few clinically or commercially successful applications. Repertoire cloning is set to overcome this problem. iii) The time period for generation of a monoclonal antibody should be much reduced compared to *in vivo* immunisation. iv) It has allowed the generation of monoclonal antibodies against antigens to which monoclonal antibodies cannot be produced *in vivo* such as the protein BiP (Nissim, 1994) and it has been claimed that antibodies with greater specificity and affinity can be generated *in vitro* than *in vivo* (Hudson, 1998). v) Most importantly, screening strategies at the end of the selection stage can be chosen, so that only those ScFvs capable of folding correctly and being secreted into the periplasm or culture broth are isolated. This is a notable advantage over the engineering of ScFvs from hybridomas, where one cannot know *a priori* whether it will fold correctly or form an inclusion body.

A word of caution may be warranted. Cloned repertoires are completely artificial and the extent to which they may suffer from polyreactivity is uncertain (Aujame *et al.*, 1997).

1.4 Intracellular expression of antibody binding sites (intrabodies) for phenotypic knockout

Studies have demonstrated that in eukaryotic cells, linear contiguous stretches of amino acids referred to as signal sequences are used to direct proteins to their final intracellular location. In the past ten years it has been empirically determined, that by transfecting genes encoding signal sequence – antibody binding site fusion proteins into cells, fully functional binding activities can be expressed in a variety of sub-cellular sites. The binding molecule, then, is referred to as an intrabody (Chen *et al.*, 1994) and can interfere with the activity of its corresponding antigen (reviewed by Biocca and Cattaneo, 1995; Cattaneo and Biocca, 1997; Marasco, 1997).

The concept of the product of one gene (say an intrabody) being used to artificially down-regulate *in trans* the activity of a second gene (say that encoding the corresponding antigen) is referred to as "phenotypic knockout" or "intracellular immunisation" (Baltimore, 1988; Cattaneo and Biocca, 1997) (see Fig. 1.9). This concept is of particular importance as applied to eukaryotic cells. Classical means of genetically manipulating cells such as random mutagenesis of the genome are not applicable to eukaryotic cells due to polyploidy and the recessive nature of most mutants. Although genotypic knockout by homologous recombination is feasible it is not generically applicable and is not amenable to the generation of conditional mutants.

Phenotypic knockout is one approach for investigating the *in vivo* function of an isolated eukaryotic gene or protein (proposed by Herskowitz, 1987). Phenotypic knockout can also be used to genetically engineer plants with desirous properties such as increased disease resistance (Tavladoraki *et al.*, 1993), delayed ripening (Hamilton *et al.*, 1995), modified colour (deLange *et al.*, 1995) and low fat content (Knauf and Facciotti, 1995). Similar approaches could be used to engineer improved lifestock (Muller and Brem, 1996; Staeheli, 1991). However, the greatest enthusiasm has been generated by the idea that phenotypic knockout approaches could be applies as gene therapies against viral diseases such as AIDS (Anderson, 1994) or genetic diseases such as cancer (Gottesman, 1994).

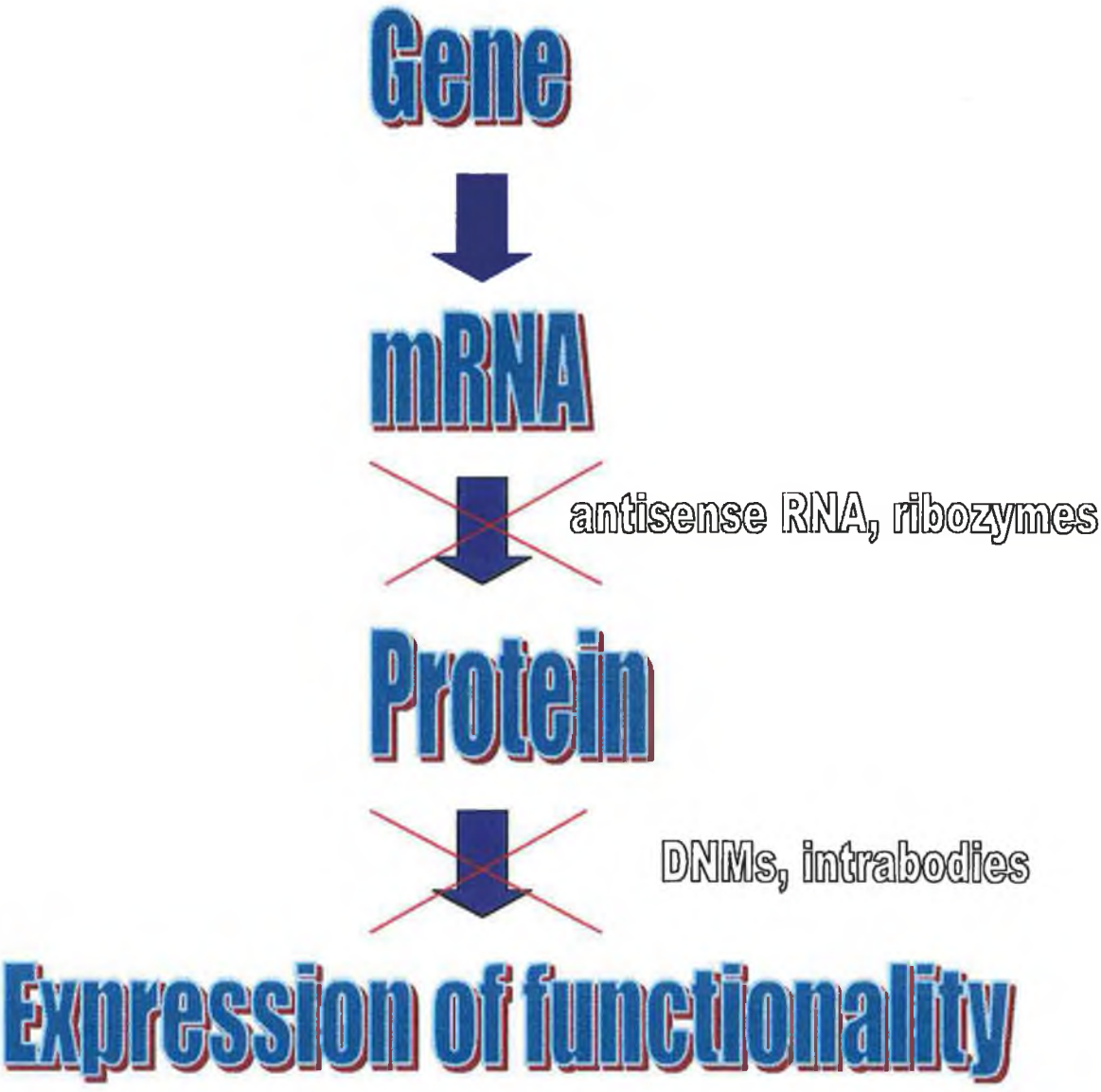


Fig. 1.9: *The flow of genetic information (central dogma of molecular biology) is depicted above. The crosses indicate the stages at which this flow can be subverted by trans-regulating gene products. Intrabodies subvert this flow by binding to the protein. This may block the proteins active site or, perhaps, retain the protein in the wrong sub-cellular compartment. The net result is the same. The protein cannot express its functionality. See section 1.4.2 for a description of the modes of action of antisense RNA, ribozymes and dominant negative mutants (DNMs).*

In the following sections the 'proof-of-principal' experiments indicating ectopic expression of functional antibody binding sites are reviewed. Intrabodies are compared and contrasted with the other common gene products used for phenotypic knockout. Some examples of intrabodies being used for this purpose are reviewed. **Finally, it is proposed that intracellular expression of diverse intrabody libraries may be of use in analysing the genotype or proteins involved in the expression of a specific phenotype.**

1.4.1 **Intrabody 'proof-of-principal' experiments:** Antibody synthesis in lymphoid cells of the B cell lineage is a tightly regulated process. It involves folding of heavy and light chains, assembly of these chains and possibly oligomerisation (reviewed by Cattaneo and Biocca, 1997a). These events are catalysed by a family of chaperone molecules in the endoplasmic reticulum (ER). Thus, it was by no means certain that antibodies, or derivatives thereof, could assemble correctly in any sub-cellular compartment other then the ER or in any cells other then the B cell.

Cattaneo and Neuberger (1987) first demonstrated that antibody molecules could assemble in cells of non-lymphoid origin. They showed that non-lymphoid cells, such as gliomas, when transfected with antibody μ and light chains could efficiently assemble and secrete polymeric IgM.

Intracellular expression (other then in the ER) was first demonstrated in yeast. Nascent antibody heavy and light chains enter the exocytic pathway by virtue of their n-terminal hydrophobic signal sequence, which is subsequently cleaved. Carlson (1988) genetically engineered the light and heavy chain genes of an anti-alcohol dehydrogenase 1 antibody so that the signal sequences were deleted. By default, proteins without any signal sequence are expected to remain in the cytoplasm (the sub-cellular location of alcohol dehydrogenase 1). Transfectants expressing both chains did show limited neutralisation of alcohol dehydrogenase 1 activity. This proved that fully functional antibody could assemble in the cytosol.

Cytosolic expression of antibodies in mammalian cells was first achieved by Biocca *et al.* (1990). Site-directed mutagenesis was used to generate a hydrophilic leader sequence from the wild type, on both a heavy and light chain.

The mutated heavy and light chains were transfected into a variety of cell lines. Cytosolic expression of functional antibody was demonstrated by immunofluorescence using an anti-idiotypic Mab. The same paper described the nuclear localisation of assembled antibody, which was achieved by replacing the mutated leader sequence of the light chain with a nuclear localisation sequence (NLS). Similarly Werge *et al.* (1990) used a NLS to redirect an antibody heavy and light chain away from the ER. When the heavy and light chain constructs were co-transfected into COS cells, immunofluorescent analysis revealed staining patterns from predominantly nuclear to predominantly cytoplasmic. These studies, however, did not demonstrate functional inactivation of a cytosolic protein due to the presence of the intrabody. Proof that this was possible was not available until 1993 when Biocca *et al.* (1993) injected mRNA encoding a leaderless ScFv into *Xenopus* oocytes. The ScFv was derived from an anti-$p21^{ras}$ antibody. Insulin-dependent meiotic maturation of the oocyte, a $p21^{ras}$-dependent process was inhibited in the injected oocytes. This was also the first demonstration that ScFvs could fold properly in mammalian cytosol. Biocca *et al.* (1994) then demonstrated by immunofluorescence that the ScFv co-localised with $p21^{ras}$ at the cytoplasmic face of the plasma membrane. Using the same ScFv construct, it was shown that T-cell receptor-mediated activation of the Jurkat cell line (dependent upon $p21^{ras}$) was inhibited by the cytosolic ScFv (Werge *et al.*, 1994).

Retention of a ScFv in the lumen of the ER was achieved by Marasco *et al.* (1993). They found that a ScFv containing a wild-type leader sequence was retained in the ER by immunoglobulin-binding protein (BiP). Surprisingly addition to the C-terminus of the ER retention sequence SEKDEL adversely affected the expression level of the ScFv. This cannot be a general characteristic of ScFvs as Biocca *et al.* (1995) demonstrated that this sequence was very useful for active retention of a ScFv in the ER lumen. In the same paper it was reported that replacing the leader sequence of a ScFv with the n-terminal presequence of the mitochondrial protein, cytochrome C oxidase, was sufficient to target the ScFv to the mitochondria. Finally, Chesnut *et al.* (1996) have demonstrated that a ScFv with a wild-type leader sequence and a mutated μ-

chain transmembrane domain can be targeted to the extracellular face of the plasma membrane.

Antibodies have also been expressed in plant cells. Hiatt *et al.* (1989) generated tobacco plants, expressing full-length heavy and light chains, and showed that fully functional antibody could assemble in the tobacco leaf. The location of the antibody was not determined however. They did find that the leader sequence was required for good expression levels to be obtained. Similar results were obtained by Benvenuto *et al.* (1991) using a V_H domain. Surprisingly, when the mammalian leader sequence was replaced with a plant leader sequence the antibody molecule was targeted to the chloroplast (During *et al.*, 1992). Owen *et al.* (1992) determined that no leader sequence was required for expression of a ScFv in plants. Again the location of the ScFv was not determined. Nonetheless, it could bind to the plant regulatory photoreceptor protein phytochrome. Seeds from transgenic progeny displayed aberrant phytochrome-dependent germination. Tavladoraki *et al.* (1993) designed a leaderless ScFv against artichoke mottled crinkle virus which they assumed was expressed in the cytoplasm. Regardless of the truth of this statement, transgenic tobacco plants showed a reduction in infectious incidents compared to wild type tobacco.

These studies demonstrate the feasibility of the technology although it is equally obvious that targeting is still not an exact science and appears to be dependent to some extent on the specific variable region. It is equally obvious that the expression levels achieved have varied with the precise antibody, its design and its sub-cellular location. The sub-cellular location is crucial. The worst location appears to be the cytoplasm. This is at least partially due to the redox state of the cytoplasm. Biocca *et al.*, (1995) demonstrated that ScFvs targeted to the cytoplasm accumulate in a reduced form while those targeted to the ER lumen or the mitochondria were oxidised. Variable domains of antibodies are known to be relatively unstable and in general the disulfide bond is required to stabilise the tertiary structure. The antibody fragment utilised is equally important. Whole molecules, Fabs (e.g. Chen *et al.*, 1994), ScFvs and V_H domains have all been used. ScFvs appear to be the format of choice because of its simpler assembly requirements. Certainly whole antibodies cannot accumulate in the cytoplasm of plant cells although it appears that ScFvs

can. Furthermore, there is some evidence that in mammalian cells, whole antibodies are proteolytically cleaved to Fvs (Biocca *et al.*, 1990). Increased knowledge of trafficking signals (reviewed by Biocca and Cattaneo, 1997b; Franconi *et al.*, 1997) should lead to ScFvs being targeted to other sub-cellular compartments.

1.4.2 Other strategies for phenotypic knockout:

*1.4.2.1 **Antisense RNA***: This topic has been extensively reviewed (see e.g. Weintraub *et al.*, 1985; Walder, 1988; van der Krol *et al.*, 1988; Helene and Toulme, 1990). Briefly, genetic regulation by antisense RNA is a naturally occurring phenomenon utilised by mobile genetic elements, by virus' and by prokaryotes (Simons and Klechner, 1988). Plasmid copy number, conjugative transmission, bacteriophage replication and expression of a number of bacterial proteins are dependent upon antisense regulation. Antisense transcripts are also found in eukaryotic cells (van der Krol *et al.*, 1988; Helene and Toulme, 1990).

Antisense RNA inhibits the flow of genetic information by binding to RNA transcribed from the gene to be regulated. Quite how binding inhibits gene expression is not certain. It could conceivably be due to premature transcript termination, translation arrest, and induced degradation of mRNA by nucleases acting upon double-stranded RNA or irreversible modification of mRNA (Helene and Toulme, 1990).

The design of an artificial antisense gene to regulate a specific gene of interest is a simple task once the gene sequence of interest is known, as the most obvious structural feature of an antisense molecule is its complementarity to the mRNA of interest. Although the design of an antisense molecule is a facile task, the *a priori* design of the most efficacious molecule is not possible at present. Instead, the random generation of antisense molecules based on the gene sequence of interest, followed by expression selection for most efficient *trans*-regulation may be a far superior approach (see Gudkov *et al.*, 1993 and section 1.4.4.1).

1.4.2.2 ***Ribozymes:*** The biology, chemistry and potential applications of ribozymes have been reviewed extensively in the recent past (see e.g. Sullivan, 1994; Christoffersen and Marr, 1995; Kashani-Sabet and Scanlon, 1995; Kiehntopf *et al.*, 1995). Ribozymes are catalytic RNA molecules with endoribonuclease activity. They occur naturally in a variety of biological systems, viral, prokaryotic and eukaryotic (single and multicellular organisms) where they cleave (in *cis* or *trans*) specific single- or double-stranded RNA molecules.

Ribozymes basically consist of a binding arm(s) and a catalytic domain. Utilising the antisense principal the binding arm binds specifically to the target RNA substrate and aligns the catalytic domain with a suitable cleavage site.

Two naturally occurring ribozymes, the hammerhead and the hairpin are quite small and have a relatively simple secondary structure. This has facilitated the design of artificial ribozymes targeted to cleave any target RNA of choice and hence to *trans*-regulate any gene of interest. This is achieved by simply mutating the binding arm so that it is complementary to the target RNA.

While antisense molecules and ribozymes are targeted in a similar fashion their mode of actions differ completely. This has one important consequence. Because ribozymes are catalytic, unlike antisense molecules they do not have to be expressed in stoichiometric amounts to the target in order to efficiently down-regulate the gene.

1.4.2.3 ***Dominant negative mutants***: As implied in section 1.4, most negative (loss-of-function) mutants are recessive in polyploid cells. However, a number of naturally occurring negative mutants are dominant over the wild type (reviewed by Wilkie, 1994; Feinberg and Trono, 1992). For example, certain human diseases such as epidermolysis bullosa simplex (Kappel *et al.*, 1994) are due to dominant negative mutants. The population dynamics and course of infection of certain viral species also appears to be determined by dominant negative genomes (Feinberg and Trono, 1992).

There are a number of ways in which dominance can be exerted. One requires that the wild type activity of a protein is dependent upon two or more discrete functional domains that can be repressed independently of one another. Mutants bearing only a subset of the functional domains could exert dominance by competing with the wild type for vital cellular factors or substrate. Thus,

Herskowitz (1987) has proposed that gene fragments generated from a gene of interest may encode dominant negative mutants. He further proposed that dominant negative mutants generated in this fashion might be used as a general method to down-regulate any gene of interest. As mentioned with regard to antisense molecules, while the *a priori* design of optimal genes is feasible in certain instances (Christian *et al.*, 1991), random approaches to the generation may be preferable (Gudkov *et al.*, 1993).

1.4.2.4 Comparison of strategies for phenotypic knockout:

	Antisense RNA	Ribozyme	Dominant negative mutant	Intrabody
Inhibition at level of:	RNA	RNA	Protein	Protein
Generated based on gene sequence?	Yes	Yes	Yes	No
Generation requires purified protein?	No	No	No	Yes
Turnover	1	multiple	1-multiple	1

Table 1.2: *A comparison of the four general classes of trans-regulating gene products is presented. Turnover refers to the number of molecules of RNA/protein inhibited per molecule of inhibitor.*

The four major classes of *trans*-regulating gene products are compared in the above table. Simply based on frequency of appearance of terms in the science citation index, antisense strategies are the preferred strategy. Ribozymes are a more recent development but the higher turnover of ribozymes may eventually make this approach more attractive than the antisense approach. In relative terms, dominant negative mutants and intrabodies are relatively

unknown quantities. Some successful applications of intrabodies are reviewed in the next section.

1.4.3 Practical applications of intrabodies:

1.4.3.1 Gene therapy for AIDS: Until recently conventional chemotherapeutic interventions have proved disappointing, as have vaccinations, for curing/preventing AIDS. For this among other reasons, this viral infection has been an attractive target for gene therapeutic intervention. HIV-1, the etiologic agent of AIDS, is a retrovirus with a complex lifecycle (reviewed by Trono, 1994). It was suggested, that down-regulation of certain key viral regulatory or structural proteins may be an efficient approach, to provide target cells with long-term protection. Initial pre-clinical studies have included the intrabody approach with a significant degree of success.

A series of studies have been carried out by Marasco's group utilising intrabodies generated from an anti-gp120 antibody referred to as F105. gp120 is an HIV-1 structural protein located on the virion surface. It is initially synthesised as a gp160 precursor, translocated into the ER, cleaved in the golgi complex to the mature gp120 and then transported to the cell surface for assembly into a viral particle. gp120 facilitates entry of the viral particle into CD4+ cells by binding to this cell surface molecule. When expressed on the cell surface, gp120 can induce cell fusion, and hence death, by binding CD4 on adjacent cells (syncythia formation). Marasco *et al.* (1993) targeted a single-chain Fv intrabody (ScF105) to the endoplasmic reticulum in an attempt to prevent gp120 being transported to the cell surface. As expected in stably transfected cell lines there was a decrease in syncythia formation and also in the infectivity of HIV-1 particles released by infected cells. They then modified this approach in order to gain the benefit of both intra- and extracellular immunisation (Chen *et al.*, 1994). While ScF105 was retained in the ER the corresponding Fab construct was efficiently secreted from stable transfectants. This allowed neutralisation of gp160 intracellularly and gp120 extracellularly. Based on these results a pre-clinical trial was approved in the U.S.A. in 1995 utilising ScF105 (Marasco, 1997).

A number of regulatory proteins including tat, rev and reverse transcriptase (RT) have also been targeted by intrabodies. Rev is a small protein that interacts in the nucleus with cellular cofactors to allow cytoplasmic accumulation of viral mRNA ultimately leading to viral replication. Duan *et al.* (1994) targeted a ScFv intrabody with anti-rev activity to the cytoplasm. The rational for this approach, was to obstruct nuclear transport of rev and hence inhibit viral replication. They found that in stable transfectants viral replication was reduced by >99% and syncythia formation was reduced drastically. Similar results were obtained by Mhashilkar *et al.* (1995) using intrabodies targeting the tat protein. Tat is a potent transcriptional activator of the HIV long terminal repeat. Productive infection by HIV-1, of cells stably expressing a ScFv-C_κ (kappa light chain constant domain) fusion protein was dramatically reduced.

In all the examples mentioned above, post-integration steps in the HIV-1 life cycle were targeted. Working on the reasonable assumption that targeting a pre-integration step would be more beneficial, Maciejewski *et al.* (1995) targeted the RT protein. In MOLT-3 cells expressing cytoplasmic anti-RT Fab, propagation of HIV-1 was completely inhibited for the monitored period of five weeks.

*1.4.3.2 **Gene therapy for cancer***: Cancer, a multifactorial genetic disorder, in many instances is refractory to conventional chemo- and radiotherapy (Kipps, 1993) and hence has become a target for gene therapies. Among the many approaches being pursued is the down-regulation of genes known to be critical for maintenance of the transformed phenotype.

Over-expression of the tyrosine kinase receptor, erbB-2 is important in the pathogenesis of a variety of neoplasms including those of the breast, lung and stomach. DeShane *et al.* (1996) attempted to inhibit transport of erbB-2 to the cell surface. To do so they generated gene constructs encoding an anti-erbB-2 ScFv targeted to either the cytosol of the ER. The ER targeted ScFv was not surprisingly the more successful. In fact it was cytotoxic for a variety of erbB-2 over-expressing cell lines due to induction of apoptosis. Of note it was more successful then an antisense molecule which was only cytostatic. Similar but not identical results were reported by Beerli *et al.* (1994).

The high affinity interleukin-2 receptor (IL-2Rα) is constitutively up-regulated in certain T- and B-cell malignancies. Richardson *et al.* (1995) have postulated that its down-regulation may be of clinical benefit. To this end they engineered an anti-IL-2Rα ScFv with the SEKDEL ER retention signal (sFvTacKDEL). The ability of sFvTacKDEL to down-regulate IL-2Rα was assessed in Jurkat cells which do not express IL-2Rα under normal circumstances but can be induced to do so by treatment with phorbol 12-myristate 13-acetate. 16 out of 16 independent clones stably transformed with the intrabody showed complete inhibition of IL-2Rα induction. It was demonstrated that IL-2Rα was retained in the ER.

Not all strategies aimed at down-regulation have proved successful. Beerli *et al.* (1994) attempted to retain the epidermal growth factor receptor (EGFR) in the ER by targeting an anti-EGFR ScFv to the lumen of the ER but only a 9% reduction in expression of EGFR was achieved.

1.4.4 Speculations on the intracellular expression of intrabody libraries

In all the examples quoted so far, intrabodies have been genetically engineered from hybridoma cell lines. It is only recently that ScFvs selected from phage display libraries have been used as intrabodies (Gargano and Cattaneo, 1997). The successful results reported may provide the impetus to directly clone immune repertoires for intracellular expression (intrabody libraries). Conceptually and technologically, this task is no more challenging then the generating phage display libraries. In this section two applications of intrabody libraries are presented.

*1.4.4.1 **'Binding selection' from intrabody libraries***: As noted in section 1.4.1, the effectiveness of an individual intrabody depends in an unpredictable fashion on its primary sequence. Hence, a ScFv selected from a phage display library, based on its binding activity *in vitro*, may not function at all well *in vivo*. It would therefore be advantageous to include a selection step *in vivo* when isolating a ScFv for use as an intrabody. Thus, Gargano *et al.* (1997) have proposed that initially, one could carry out panning of a phage display library to generate a relatively small library of ScFvs enriched for the desired reactivity.

One could then utilise the yeast-two-hybrid system to select ScFvs, which bind the antigen *in vivo*. The two-hybrid system provides an experimental system in yeast cells, to monitor intracellular protein-protein interactions. It will not be discussed here but is thoroughly reviewed by Fields and Sternglantz (1994) and Finlay and Brent (1995). Suffice it to say that the enriched polyclonal ScFv population could be engineered as 'bait' and the antigen engineered as 'prey'. A subsequent screen would allow isolation of a ScFv with good intracellular binding properties.

To a certain extent, the technology for isolation of genetic suppressor elements (GSEs) for a specific gene can be seen as a forerunner of the above technique. GSE is the name given by Roninson's group to "short biologically active gene fragments that encode dominantly acting peptides or inhibitory antisense RNAs" (Roninson *et al.*, 1995). Just as one cannot guarantee the effectiveness of an intrabody, an antisense molecule or dominant negative mutant generated from a specific gene cannot be guaranteed to be effective *in vivo*. In order to isolate GSEs for a specific gene, it was proposed that one create a library of random gene fragments from the gene of interest. GSEs are then selected by cloning the resulting library into a relevant cell host followed by biological selection of clones conferring the phenotype associated with suppression of the target. Using this strategy Gudkov *et al.* (1993) have isolated GSEs for topoisomerase II.

1.4.4.2 'Phenotypic selection' from intrabody libraries: **The idea of selecting intrabodies *in vivo* from relatively small biased intrabody libraries could be brought to its logical conclusion by selecting intrabodies *in vivo* from large unbiased intrabody libraries, a strategy referred to as 'phenotypic selection' by Gargano *et al.* (1997).** Why this strategy is worth pursuing is best illustrated with a concrete example. Consider the AIDS virus. Intrabodies are considered to have considerable therapeutic potential against this cytotoxic virus. Attempts to generate therapeutically relevant intrabodies have so far revolved upon the *a priori* isolation of specific antibodies considered to have therapeutic potential, followed by their engineering as intrabodies and subsequent evaluation (see section 1.4.3.1). This rational strategy requires the following:

i) that the biochemistry of the virus is understood in sufficient detail that a critical protein can be chosen, in order to isolate a specific ScFv from a phage display library

ii) that the protein chosen is available in sufficient quantity and purity

iii) that the engineered intrabody is of sufficient stability

iv) that binding of the intrabody to the target protein actually does interfere with the function of the protein.

No such problems pertain to the random strategy of phenotypic selection. This strategy involves cloning an entire intrabody library into a relevant cell host. Subsequently, the transfected population of cells can be challenged with the AIDS virus. Natural selection should then result in the isolation of intrabodies conferring resistance (for whatever reason) to the virus, should the initial library contain such intrabodies.

Looked at from a different perspective, the usefulness of this concept is even more apparent. **While the rational approach results in the *in vivo* screening of only a tiny fraction of the phage display library, the random approach allows screening of the entire library for anti-viral activity.**

Again this concept was foreseen and was applied for the isolation of GSEs conferring a desired selectable phenotype (Roninson *et al.*, 1995). Although introduced by way of a specific example, this strategy should be of use wherever a selectable or screenable phenotype is involved. In particular, phenotypic selection may be a very useful tool for functional genomic analyses. It has already been mentioned (section 1.4), that down regulation of a specific gene may be one approach to analysing its *in vivo* function. One would simply generate a cell line in which the gene of interest is down-regulated. Comparison of the phenotypes expressed by the engineered cell line with the wild-type should provide information on the functionality of the gene. In effect this strategy links phenotypes to a specific gene.

Two potential problems may hamper application of this paradigm. Firstly, assuming a phenotypic change occurs upon down-regulation of the gene, if one has no idea at all in advance as to the potential role of the gene, detection of the change may be very difficult. Secondly, and perhaps more importantly, down-regulation of many genes may not result in any phenotypic changes whatsoever due to the degeneracy in protein function. If the protein encoded by

the gene does not occur at a crucial regulatory step in its biochemical pathway the cell may be able to compensate for its absence and express the same phenotypes as the wild-type.

One suggestion is that, these problems could be circumvented, by inverting the paradigm and linking proteins (genes) to a specific phenotype. Phenotypic selection is admirably suited to this task. For example, if one wanted to isolate proteins involved in programmed cell death, one could clone an intrabody library into a relevant cell host, chemically induce apoptosis, and let natural selection enrich for intrabodies preventing apoptosis. The cellular proteins bound by enriched intrabodies are then presumed to be part of the biochemical pathways involved in apoptosis. Furthermore, they presumably occupy critical positions in the pathways. As another example one could utilise FACS as a high thoughput screen to isolate intrabodies targeting proteins involved in cell proliferation (see Fig. 1.10). In general, as long as a biological selection or a high throughput screen is available, intrabodies can be isolated conferring any phenotype you desire.

Such phenotypic selection strategies will be dependent upon:

i) generating very large intrabody libraries (to increase the average affinity of the library for any randomly chosen protein)

ii) increased understanding of the causes of intrabody instability so that generic protein engineering solutions can be found to increase the intracellular concentration of functional intrabody

iii) cloning the library into as small a population of cells as possible.

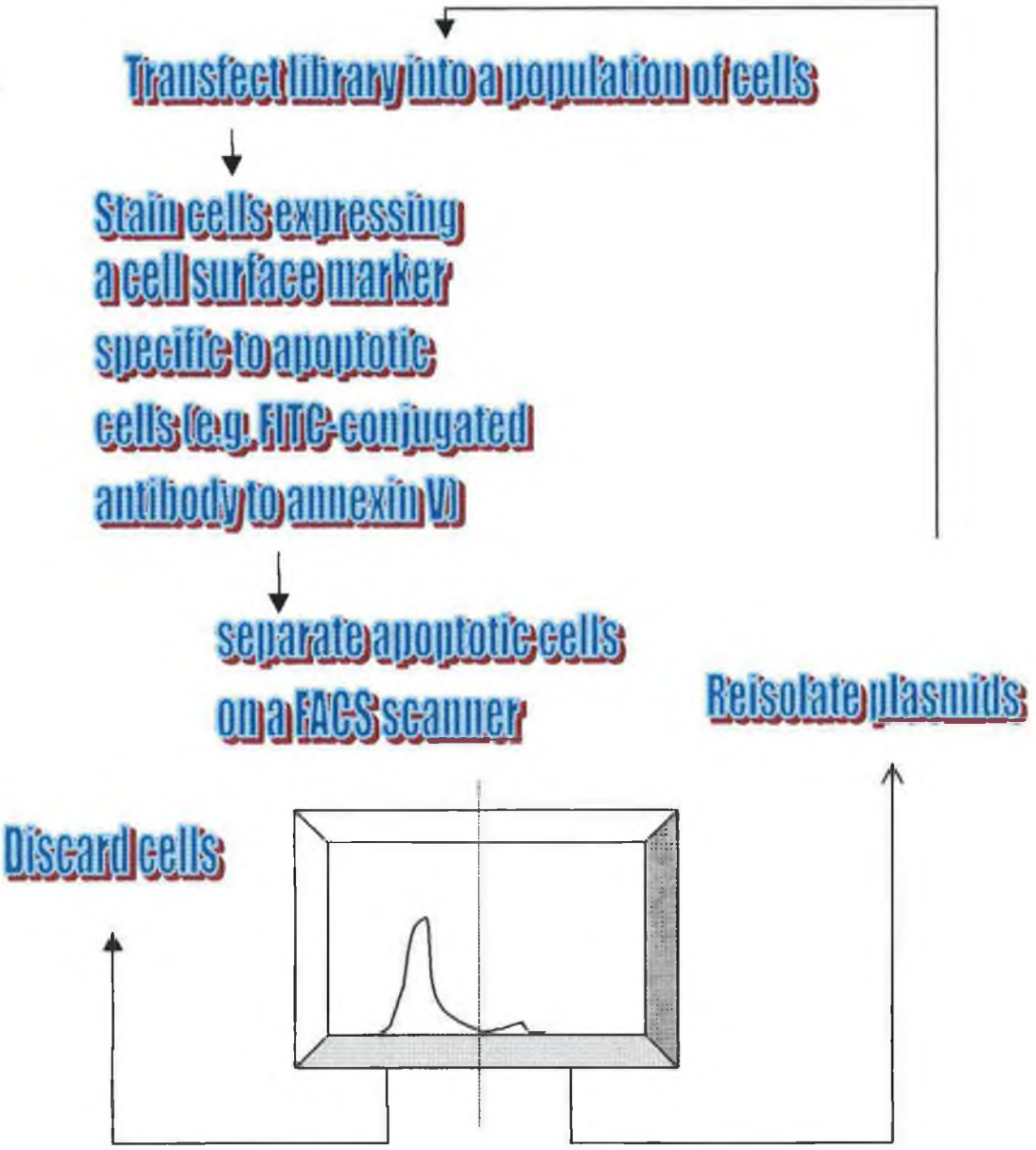


Fig. 1.10: *A flow diagram suggesting a means of isolating apoptosis-inducing intrabodies. The intrabody library is transfected into a relevant cell population. After a suitable time period has elapsed (say 24hrs) the cells are screened using a FACS and an antibody specific for annexin V. This is a component of the plasma membrane, normally residing at the cytoplasmic face. It is translocated to the external face, however, in apoptotic cells. The above schematic of a computer screen, shows cell numbers along the y-axis and fluorescent intensity along the x-axis. Only those cells fluorescing above a certain intensity are harvested. The plasmids they harvest are re-isolated by PCR, or transfection into E. coli. This sub-library can subsequently be rescreened.*

1.5 Aims

One area of interest was the nature of antibody binding sites and, in particular, the concept that they may be polyreactive. Specifically, it was suggested that IVI (section 1.3.2.2) would produce polyreactive Mabs. In order to test this hypothesis it was decided to produce a panel of Mabs, by IVI, and to use a number of assays to determine whether they showed a polyreactive binding profile. These studies are reported in chapter three.

A second area of interest was the use of intrabodies for phenotypic knockout. In particular, interest focused on the generation of intrabody libraries because of their potential for use as functional genomic tools or as sources of therapeutic proteins. These studies are reported in chapters four and five.

CHAPTER 2

MATERIALS AND METHODS

2.1 Equipment

Class	Model	Source
Biacore™	Biacore™ 1000	Pharmacia Biosensor
Centrifuges	Heraeus Christ Labofuge 6000 Biofuge A Microcentrifuge Sorvall refridgerated centrofuge	Heraeus Instruments Inc. Heraeus Instruments Inc. Du Pont Instruments
CO_2 Tissue culture incubator	Jouan EG 115 IR	Jouan
Cytospin	Heraeus Labofuge Ae	Heraeus Instruments Inc.
Electroporator	Gene Pulser Electroporator	Bio-Rad Labs.
Microscope	Leitz Laborlux K fluorescence microscope Nikon Diaphot inverted microscope	Laboratory Instruments and Supplies Micron Optical Co. Ltd.
Miniblotter	Miniblotter MN100-25	Immunetics
Orbital Incubator	Gallenkamp INR-201-010P	AGB
Protein Electrophoresis Apparatus	Atto dual minislab system AE-6450	Atto Corp.
Protein Electrotransfer Cell	Bio-Rad Transblot cell	Bio-Rad Labs.

Rocker Platform	Stuart Platform Shaker STR6	Lennox
Spectrophotometers	U.V.-160A Spectrophotometer	Shimadzu Corp.
	Titertek Multiscan Plate Reader	Flow Lab. Ltd.
Thermocycler	LEP PREM™	Medical Supply Co.
Ultrafiltration Cell	Stirred Cell 8400	Amicon Inc.

AGB: Dublin Industrial Estate, Dublin 11, Ireland.

Amicon Inc.: Beverly, Massachusetts 01915, U.S.A.

Atto Corp.: 2-3 Hongo 7-Chrome, Bunkyo-Kui, Tokyo 113, Japan.

Bio-Rad Labs.: 3300 Regatta Boulevard, Richmond California 94980, U.S.A.

Du Pont Instruments: Instrument Products Division, Newtown, Connetticut 06470, U.S.A.

Flow Labs. Ltd.: Woodcock Hill, Harefield Rd., Richmansworth, Hertfordshire WD3 1PQ, England.

Heraeus Instruments Inc.: 111-a Corporate Boulevard, South Plainfield New Jersey 07080, U.S.A.

Immunetics: 380 Green St., Cambridge, Massachusetts 02139, U.S.A.

Jouan: Zone Industrialle de Brais, Saint Nazaire, France.

Laboratory Instruments and Supplies: Ashbourne, Co. Meath, Ireland.

Lennox: P.O Box 212A, John F. Kennedy Dr., Naas Rd., Dublin 12, Ireland.

Medical Supply Company: Damestown, Mulhuddart, Dublin 15, Ireland.

Micron Optical Co. Ltd.: Bray, Co. Wicklow, Ireland.

Pharmacia Biosensor: St. Albans, Hertfordshire AL1 3AW, England.

Shimadzu Corp.: 1 Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto 604, Japan.

2.2 Consumable items

Class	Item	Source
Bacteria	*E. coli* DH5α containing pCH110	Dr. Barbara Fingleton
	Electrocompetent *E. coli* XL1-Blue	Dr. Tony Killiard
	Electrocompetent *E. coli* XL1-Blue MRF'	Stratagene Ltd.
	Nissim Library	Medical Research Council
Hardware	CM5 Chips	Pharmacia Biosensor
	Electroporation cuvettes	Bio-Rad Labs.
	Nitrocellulose	Sigma Chemicals
	Nunc Maxisorp plates	Nunc
	Tissue Culture plastic-ware	Corning Costar
	Ultrafiltration membranes	Amicon Inc.

Amicon Inc.: Beverley, Massachusetts 01915, U.S.A.

Bio-Rad Labs.: 3300 Regatta Boulevard, Richmond, California 94980, U.S.A.

Corning Costar: High Wycombe, Buckinghamshire HP13 6EQ, England.

Drs. Barbara Fingleton and Tony Killiard: Dublin City University, Dublin 9, Ireland.

Medical Research Council: Centre for Protein Engineering, Medical Research Council Centre, Hills Rd., Cambridge CB2 2QH, England.

Nunc: P.O. Box 280-Kamstrup DK, Roskilde, Denmark.

Pharmacia Biosensor: St. Albans, Hertfordshire AL1 3AW, England.

Sigma Chemicals: Fancy Rd., Poole, Dorset BH12 4QH, England.

Stratagene Ltd.: 140 Cambridge Science Park, Milton Rd.,Cambridge CB4 4GF, England.

2.3 Reagents and Chemicals

All chemicals were reagent grade and were purchased through **Sigma Chemicals** except as noted below.

Class	Chemical	Supplier
Biochemistry	Alkaline phosphatase-labeled antibodies	Southern Biotechnology
	anti-CD5, clone E51.87	Seikagaku Corp.
	anti-lipopolysaccharide, clone11E10	Southern Biotechnology
	normal mouse IgM	Southern Biotechnology
Microbiology	Agar	Oxoid
	Terrific medium	Oxoid
	Tryptone	Oxoid
	Yeast extract	Oxoid
Molecular Biology	*Eco*RI	Boehringer Mannheim
	*Eco*RV	Boehringer Mannheim
	Equilibrated phenol	Oncor-Appligene
	Eukaryotic TA-cloning kit containing pCR™3-Uni	Invitrogen
	*Hin*dIII	Boehringer Mannheim
	Nucleotides	Promega Corp.
	pcDNA3.1+	Invitrogen
	PCR 'Clean-up' kit	Boehringer Mannheim
	PCR Primers	Genosys
	Pwo polymerase	Boehringer Mannheim
	Qiagen gel extraction kit	Qiagen
	Qiagen maxiprep kit	Qiagen
	RNase1	Boehringer Mannheim
	Synthetic duplex DNA	Genosys
	Taq polymerase	Promega Corp.

	T4 DNA polymerase	Boehringer Mannheim
	T4 polynucleotide kinase	Boehringer Mannheim
Tissue culture	Briclone	BioResearch Ireland
	Dulbecco's modification of Eagles medium	Gibco BRL
	Fetal calf serum	Gibco BRL
	HEPES	Gibco BRL
	Hybridoma SFM™	Gibco BRL
	Iscoves modification of Eagles medium	Gibco BRL
	L-glutamine	Gibco BRL
	Non-essential amino acids	Gibco BRL
	Oleic acid	Aldrich Chemical Co.
	Palmitic acid	Aldrich Chemical Co.
	Phosphate-buffered saline tablets	Oxoid
	Rabbit serum	Gibco BRL
	Sodium pyruvate	Gibco BRL
	Tfx™	Promega
	Vectashield mounting medium	Vector Labs.

Aldrich Chemical Co.: Old Brickyard, New Rd., Gillingham, Dorset SP8 4JL, England.

BioResearch Ireland: Glasnevin, Dublin 9, Ireland.

Boehringer Mannheim: Bell Lane, Lewes, East Sussex BN7 1LG, England.

Genosys: 162A Cambridge Science Park, Milton Rd., Cambridge CB4 4GH, England.

Gibco BRL: Trident House, Renfrew Rd., Paisley PA4 9RF, Scotland.

Invitrogen: De Schelp 12, 9351 VV, Leek, Netherlands.

Oncor-Appligene: Chester-le-Street, Durham DH3 2TD, England.

Oxoid: Basingstoke, Hampshire, England.

Promega Corp.: 280 Woods Hollow Rd., Madison, Wisconsin 53711-5399, U.S.A.

Qiagen: Boundary Court, Gatwick Rd., Crawley, West Sussex RH10 2AX, England.

Seikagaku Corp.: 1-5, Nihonbashi-honcho 2-chome, Chuo-ku, Tokyo 103, Japan.

Southern Biotechnology: 160A Oxmoor Boulevard, Birmingham, Alabama 35209, U.S.A.

Vector Labs.: Burlingame, California 94010, U.S.A.

2.4 Biological Solutions

The following solutions are referred to in the text, by name only. The final concentration of constituents, in the following solutions, are given in appendix C.

2.4.1 Mammalian cell culture

Phosphate-Buffered Saline (PBS)

This always refers to Dulbecco's A PBS, 0.14M, pH 7.2-7.4. The components were obtained premixed as tablets and dissolved in the correct volume of distilled H_2O (dH_2O).

2.4.1.1 Splenocyte preparations:

Dissection Hanks Balanced Salt Solution (DHBSS)

$CaCl_2.H_2O$	00.194g
KCl	00.400g
K_2HPO_4	00.060g
$MgCl_2$	00.046g
$MgSO_4$	00.049g
NaCl	08.000g
Na_2HPO_4	00.048g
D-Glucose	01.000g
Phenol Red	00.010g

The components were dissolved in 1L of ultra-pure H_2O (UPH_2O), adding $CaCl_2.H_2O$ last, and autoclaved. The resulting solution was supplemented with gentamicin and amphotericin, to final concentrations of 50ng/ml and 2.5µg/ml, respectively. The solution was stored at -20^{0}C.

Geys Haemolytic Solution

Geys Haemolytic Solution was made up as follows not more then 30 min. before use:

dH_2O	14.6ml
Geys A	04.000ml
Geys B	01.000ml
Geys C	00.400ml

Geys A:

NH_4Cl	35.000g
KCl	01.850g
K_2HPO_4	00.111g
D-Glucose	05.000g
Phenol Red	00.050g
Gelatin	25.000g

The components were dissolved in 1L of dH_2O, autoclaved, and stored at room temperature (RT).

Geys B:

$MgCl_2$	01.970g
$MgSO_4$	00.749g
$CaCl_2.2H_2O$	04.500g

The components were dissolved in 1L of dH_2O, autoclaved, and stored at RT.

Geys C:

5.6% (w/v) solution (sterile-filtered) of $NaHCO_3$ in dH_20, stored at 4^0C.

2.4.2 Molecular biology

Tris/EDTA (TE) Buffer

This always refers to a 10mM Tris.Cl (pH 8.0) solution containing 1mM EDTA, prepared in UPH_2O, autoclaved, and stored at RT.

2.4.2.2 DNA minipreps:

Alkaline-lysis Solution 1

An autoclaved 25mM Tris.Cl (pH 8.0) solution is supplemented as follows. It is made up to 50mM glucose from a 20% (w/v) autoclaved glucose solution. It is made up to 10mM EDTA, from a 0.5M EDTA (pH 8.0) stock, and stored at 4^0C.

Alkaline-lysis Solution 2

NaOH	00.8g
Sodium Dodecyl Sulphate (SDS)	01.000g

The components were dissolved in 100ml of ultra-pure H_2O, autoclaved, and stored at 4^0C.

Alkaline-lysis Solution 3

5M $KC_2H_3O_2$	60.0ml
Glacial acetic acid	11.5ml
dH_2O	28.5ml

The components were mixed thoroughly and stored at 4^0C.

2.4.2.2 T4 DNA polymerase-based cloning of polymerase chain reaction (PCR) product:

Cut-back Buffer

Tris	00.799g
$KC_2H_3O_2$ (pH 7.9)	01.315g
$MgC_2H_3O_2.4H_2O$	00.429g
dithiothreitol (dTT)	00.154g

The components were dissolved in 100ml UPH_2O, and sterilised. This solution was then supplemented with 5ml of 2% (w/v) gelatin, and 400µl of either 100mM deoxythymidine triphosphate (dTTP) or 100mM deoxyadenosine triphosphate (dATP), for cut-back of the vector or PCR product, respectively. The buffer was stored at -20^{0}C.

Ligation buffer

Tris.Cl (pH 7.8)	01.211g
$MgCl_2$	00.095g
dTT	00.308g

The components were dissolved in 100ml UPH_2O and sterilised. 20µl of a 100mM stock of dATP was added per 1ml of solution. The buffer was stored at -20^{0}C.

2.4.2.3 Genomic DNA preparation:

PCR Lysis Solution

10x PCR Buffer (*Taq* or *Pwo*)	00.100ml
UPH_2O	00.891ml
NP-40	00.005ml
Tween-20	00.005ml

The components were mixed thoroughly, and stored at 4^{0}C.

Proteinase K

A 10mg/ml solution of Proteinase K in UPH_2O, was incubated at 37^0C for 2 hrs, and stored at -20^0C.

2.4.3 Biochemistry

2.4.3.1 Solid-phase immunoassays:

2-Amino-2-Methyl-1-Propanol (AMP) Buffer

$MgCl_2$	00.070g
Triton X-405	00.100ml
NaN_3	01.000g

The components were dissolved in 50ml dH_2O. 98.5ml of AMP was slowly added, while stirring. The volume was brought to 900ml with dH_2O and the pH adjusted to 10.25, with conc. HCl. The pH was left to equilibrate overnight and then readjusted to 10.25. The volume was brought to 1L, and stored at 4^0C.

Coating Buffer

K_2HPO_4	01.742g
KH_2PO_4	00.680g
$Na_2EDTA.2H_2O$	00.186g
NaCl	04.000g
NaN_3	00.250g

The components were dissolved in 500ml dH_2O, and stored at 4^0C.

Wash Buffer

Tris.Cl (pH 7.4)	01.211g
NaCl	08.766g
Tween 20	01.000ml
Thimerosal	00.050g

The components were dissolved in 1L of dH_2O, and stored at 4^0C.

2.4.3.2 Immunoblots and related techniques:

Coomassie Blue Stain Solution

Coomassie blue R-250	01.000g
Methanol	450ml
dH_2O	450ml
Glacial acetic acid	100ml

The components were dissolved and mixed thoroughly, and stored at RT.

Destain Solution

Methanol	100ml
Glacial acetic acid	100ml
dH_2O	800ml

The above components were mixed, thoroughly, and stored at RT.

Electrophoresis Buffer

Tris	03.000g
Glycine	14.400g
SDS	01.000g

The components were dissolved in 1L of dH_2O, and stored at RT.

Homogenising Buffer

Tris.Cl (pH 6.8)	00.363g
dTT	00.771g
EDTA	00.015g
10% (w/v) SDS	10.0ml

The components were dissolved in or mixed with sufficient dH_2O to bring the final volume to 50ml. This solution was supplemented with aprotinin to a final conc. of 1μg/ml, pepstatin to a final conc. of 1μg/ml and TLCK to a final conc. of 50μg/ml, and stored at 4^0C.

Lysis Buffer

Tris.Cl (pH 8.0)	01.211g
KCl	00.746g
$MgCl_2$	00.050g
NP-40	00.500ml

The components were mixed and dissolved in 100ml UPH_2O. This solution was supplemented with aprotinin to a final conc. of 1μg/ml, pepstatin to a final conc. of 1μg/ml and TLCK to a final conc. of 50μg/ml, and stored at 4^0C.

Sample Buffer

1M Tris.Cl (pH 6.8)	00.600ml
50% (v/v) Glycerol	05.000ml
10% (w/v) SDS	02.000ml
2-ME (2-Mercaptoethanol)	00.500ml
1% (w/v) Bromophenol blue	00.900ml

The components were thoroughly mixed together, and stored at -20^0C.

Solution A

Acrylamide	29.200g
Bisacrylamide	00.800g

The components were dissolved in 100ml dH_20, and stored in the dark at 4^0C.

Solution B

2M Tris-HCl (pH 8.8)	75.0ml
10% (w/v) SDS	04.0ml
dH_20	21.0ml

The components were thoroughly mixed together, and stored at 4^0C.

Solution C

1M Tris.Cl (pH 6.8)	50.0ml
10% (w/v) SDS	04.0ml
dH_2O	46.0ml

The components were thoroughly mixed together, and stored at 4^0C.

Transfer Buffer

Tris	03.100g
Glycine	14.400g
Methanol	200ml

The components were dissolved in 1L of dH_2O, and stored at RT.

2.4.3.3 *In situ β-galactosidase staining*

Fix Solution

25% (w/v) Glutaraldehyde	00.400ml
0.5M phosphate buffer (pH 7.3)	10.0ml
0.1M EGTA (pH 8.0)	02.500ml
1.0M $MgCl_2$	00.100ml
dH_2O	37.0ml

The components were mixed, thoroughly, and stored at RT

Rinse Solution

0.5M phosphate buffer (pH 7.3)	40.0ml
dH_2O	160.0ml
1.0M $MgCl_2$	0.400ml
Sodium deoxycholate	0.020g
NP-40	0.040ml

The components were dissolved and mixed thoroughly, and stored at RT.

Stain Solution

Rinse solution	10.0ml
5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (25mg/ml in dimethyl formamide)	0.400ml
Potassium Ferricyanide	0.0165g
Potassium Ferrocyanide	0.0165g

The components were dissolved and mixed just before use.

2.5 Mammalian cell Culture

All mammalian cell cultures were grown in a 5% CO_2 atmosphere, at 37^0C. All cell counts were made using a Neubauer Counting Chamber. Viable cell counts were obtained by mixing cells, with a 1/4 volume of a commercial 0.4% (w/v) isotonic Trypan blue solution. Dead cells were stained blue. The viable cell count was carried out within 5 min. of the addition of Trypan blue. Cells were visualised with a phase contrast microscope. All cells were pelleted by centrifugation and all centrifugations were at 200-250g for 5 min. unless otherwise stated.

2.5.1 Mammalian cell culture media:

2.5.1.1 DMEM: 500ml of 1 x DMEM (Dulbecco's modification of Eagle's medium) was supplemented with 2mM l-glutamine, 20mM HEPES and 25µg/ml gentamicin.

2.5.1.2 LINO: The following solutions were added to 500ml of IMDM (Iscove's modification of Dulbecco's medium):

0.5ml of a fresh 2.5mg/ml solution of Bovine Serum Albumin (BSA) in IMDM

0.5ml of a 0.5% (w/v) solution of bovine insulin in 0.01N HCl. This solution was prepared in advance, and stored at -80^0C.

5ml of a 0.35% (w/v) solution of human transferrin in PBS. This solution was prepared in advance, and stored at -20^0C.

100µl of each of 0.5% (w/v) solutions of palmitic acid, oleic acid and linoleic acid in absolute ethanol. Fresh preparations were used.

0.5ml of a fresh $2x10^{-2}$M solution of ethanolamine in PBS.

This medium was stored at -80^0C, and supplemented with $5x10^{-5}$ M 2-mercaptoethanol (2-ME), non-essential amino acids (NEAA) and 1mM sodium pyruvate before use.

*2.5.1.3 **HAT***: DMEM supplemented with NEAA, 1mM sodium pyruvate, 100μM hypoxanthine, 400nM aminopterin, 16μM thymidine and 10% (v/v) fetal calf serum (FCS).

*2.5.1.4 **HT***: HT medium was prepared essentially as described for HAT medium (section 2.5.1.3), but aminopterin was omitted.

2.5.2 Growth of cell lines

*2.5.2.1. **Suspension cell lines***: Cells stored in liquid nitrogen, in cryovials, were thawed at 37^0C. The cell suspension, was transferred to a 50ml centrifuge tube and 10ml of culture medium was added slowly. The cells were pelleted, and resuspended in 5ml of culture medium. The resulting cell suspension was cultured in a T-25 flask. To subculture cells, they were flushed off the surface of the flask using a pasteur pipette, into a larger volume of fresh culture medium, which was divided into the required number of flasks. For large cell quantities, cells were grown in T-75 flasks at 15ml per flask.

*2.5.2.2 **Adherent cell lines***: Adherent cell lines were grown in the same fashion as the suspension cell lines, with the exception of subculture. To subculture, culture medium was decanted from the flask and the flask was briefly washed with PBS. Cells were exposed to 1-2ml of a solution of PBS containing trypsin (0.25% (w/v)) and EDTA (0.2mg/ml), until cells were seen to detach from the plastic surface. Culture medium (10ml per ml of trypsin/EDTA) was added to the resulting cell suspension and the cells were pelleted by centrifugation at 250g for 10 min. The resulting pellet was resuspended in the required volume of fresh culture medium, which was divided into the required number of flasks.

2.5.3 Storage of cell lines

Cells were harvested, from the surface of 1xT-75 flask, by flushing or trypsinizing, and pelleted. Pellets were resuspended in 4ml of a 10% (v/v) solution of DMSO (Dimethyl sulphoxide) in FCS, and divided into 4 cryovials. A freezing-tray was used to gradually cool the cells, over a 2.5 hr. period, in the vapour-phase of liquid nitrogen. They were then immersed in the liquid-phase for long-term storage.

2.5.4 Cell lines

2.5.4.1 ***Suspension cell lines***: Sp2/0 (ATCC CRL 1581), EL4 (ATCC TIB 39) and Mycl-9E10 (ECACC 85102202). These cell lines were cultured in DMEM containing 10% (v/v) FCS. They were subcultured, using a split ratio of 1:4, at approximately 70% confluency. The term split ratio is used as defined by Freshney, 1983: "the divisor of the dilution ratio of a cell culture at subculture, e.g., one flask divided into four or 100ml up to 400ml would be a split ratio of 4."

2.5.4.2 ***Adherent cell lines***: NIH3T3 (ATCC CRL 1658) and BHK (ATCC CCL 10) were cultured in DMEM containing 10% (v/v) FCS. They were subcultured, at a split ratio of 1:6, at approximately 90% confluency. The culture medium was changed at least every second day. CHO (ATCC CCL 61) was cultured in DMEM containing 10% (v/v) FCS and NEAA, but, otherwise, was treated in the same fashion as the other adherent cell lines.

2.5.5 Conditioned media

2.5.5.1 ***Thymocyte-conditioned media (TCM)***: 4 x 3-4 week old Balb/C mice were sacrificed, by cervical dislocation, and their thymus' were removed. A thymocyte suspension was prepared, in DHBSS, by cutting the thymus' in two and gently teasing the cells out into 20ml DHBSS, through a 60 mesh metal screen. Large particulate matter was removed, by centrifuging at 80g for 30 sec.

The cells were pelleted, from the resulting suspension, and resuspended in 2ml of a 5% (v/v) solution of FCS in DHBSS. 10ml of Geys haemolytic solution was added, and the cell suspension was placed on ice for 5 min. The cell suspension was underlaid with 2.5ml of FCS and centrifuged at 300g for 10 min. The pellet was washed once in 10ml of 5% (v/v) FCS in DHBSS.

The pellet was resuspended in LINO, at $5x10^7$ cells/ml and cultured in 25ml volumes in T-75 flasks for 48 hrs. The supernatant was harvested, by centrifugation, sterile-filtered, and stored at -80^0C.

*2.5.5.2 **Mixed lymphocyte reaction (sMLR)***: Thymocytes were harvested from thymus' donated by an equal number of Balb/C and C57BL\10 mice, and erythrocytes lysed, as per section 2.5.5.1. The final cell pellet was resuspended in DMEM containing 2% (v/v) rabbit serum (RS) and 50μM 2-ME. Cells were cultured for 48 hrs in 50ml volumes in T-75 flasks. The supernatant was harvested, sterile-filtered, and stored at -80^0C.

*2.5.5.3 **EL4-conditioned media (sEL4)***: EL4 cells were routinely cultured in DMEM containing 10% (v/v) FCS. Cells were harvested, and washed using 20ml of DMEM containing NEAA, 1mM sodium pyruvate and 1% (v/v) RS. Cells were resuspended, at $1x10^6$ cells/ml, in the latter medium. Phorbol 12-myristate 13-acetate (PMA) was added, to a final concentration of 10ng/ml, from a concentrated stock in absolute ethanol. Cells were cultured for 40 hrs, in 20ml volumes, in T-75 flasks. The supernatant was harvested, sterile-filtered, and stored at -80^0C.

2.5.6 *In vitro* immunisations

*2.5.6.1 **Method of Ossendorp et al. (1986)***: The desired number of 6-8 week old Balb/C mice (approximately 1 x Balb/C per 20ml of final IVI culture) were sacrificed and their spleens removed. Nucleated splenocytes were prepared in the same fashion as were thymocytes (section 2.5.5.1), except for the fact, that splenocytes were

removed from the spleen, by flushing DHBSS through the spleen capsule with a 21g lance and a 1ml syringe.

The final nucleated splenocyte pellet was resuspended in LINO at $1x10^7$ cells/ml. An equal volume of TCM was added. Finally, the desired amount of immunogen was added, from a concentrated stock in PBS. The resulting suspension was cultured in 6 well plates at 2ml per well.

2.5.6.2 Method of Borrebaeck and Moller (1986): Nucleated splenocytes were prepared as per section 2.5.6.1. The final nucleated splenocyte pellet was resuspended, at $5x10^6$ cells/ml, in the following medium. If the final volume was to be X ml, the cell pellet was resuspended in X/3 ml of DMEM containing 10% (v/v) RS, NEAA, 1mM sodium pyruvate and 50μM 2-ME. X/3 ml of sMLR and X/4 ml of sEL4 were added and the volume was adjusted to X ml with DMEM containing 2% (v/v) RS, NEAA, 1mM sodium pyruvate and 50μM 2-ME. Antigen was added, as desired, from a concentrated stock in PBS. The cell suspension was cultured, in 10ml volumes, in T-25 flasks.

2.5.7 Hybridoma isolation

2.5.7.1 Somatic cell fusion: Sp2/O cells were grown for at least two weeks, prior to fusion. Cells were not grown above 50% confluency, and were subcultured at a split ratio of 1:2 the day before fusion. On the day of fusion, the medium was poured off the Sp2/0 cells, and DMEM lacking HEPES was added. Cells were resuspended and counted. The cells were stored on ice until required.

Splenocytes, from an *in vitro* immunisation, were resuspended and counted. Splenocytes and Sp2/O cells were mixed to give a cell ratio of 10 splenocytes per Sp2/0 cell. This cell mixture was pelleted and washed 4 times with 5mls of DMEM lacking HEPES.

All of the supernatant, from the final wash, was removed, except for 50-100μl (simply pouring of the supernatant sufficed), and the cells were resuspended by tapping. The cell suspension was placed in an ice/water bath, and 1.5ml of 50%

(v/v) PEG (polyethylene glycol) was added to it, over a 1 min. period, while swirling. Swirling was maintained for another 1.5 min. The centrifuge tube was removed, from the water bath, and enclosed in the palm of the hand. 20ml of pre-warmed (37^0C) DMEM lacking HEPES was then added over 5 min. with constant, slow, swirling. The resulting suspension was placed at 37^0C for 15-20 min.

The suspension was centrifuged, at 200g for 10 min., and the cells were resuspended at $1.2x10^6$ cells/ml, in HAT supplemented with 5% (v/v) Briclone. The suspension was plated, in 96 well plates, at 0.1ml per well, and incubated for 7 days. On day 7, 50µl of fresh HAT with 5% (v/v) Briclone was added to each well. On day 8, 50µl of spent medium was removed and fresh medium was added. Wells were then fed as required.

*2.5.7.2 **Screening for antigen-reactive wells***: Outgrown wells, from the masterplates, were screened by an ELIspot assay (2.8.1.2.). Briefly, half the cells from a well, which contained visible colonies, were placed into an eppendorf tube and washed in three volumes of fresh HAT medium. The cells, then, were resuspended in a 100µl volume of HAT medium, and used as a test sample in the ELIspot assay, as per section 2.8.1.2.

*2.5.7.3 **Cloning of antigen-reactive hybridomas***: Cells from wells, which tested positive in the ELIspot assay, were expanded in HAT medium, from 96 well plates, to 24 well plates and finally to 6 well plates.

Cells were cloned, by limiting dilution, when they had reached 50% confluency. Briefly, cells were resuspended, and diluted to 100 cells/ml in HT supplemented with 5% (v/v) Briclone. The cell suspension was plated, then, at 100µl/well in 96 well plates. After 7 days, 50µl of fresh HT containing 5% (v/v) Briclone was added, and on day 8 half the medium was changed. Wells were fed as required. Screening for positive wells proceeded, as per section 2.5.7.2, or using an ELISA as per section 2.8.1.1, using neat medium from the well, as the test sample.

Positive wells were expanded, as already described, and recloned twice at 10 cells/ml. The second time, cells were cloned in DMEM containing 10% (v/v) FCS,

NEAA and 1mM sodium pyruvate rather then in HT medium. Positive wells at this stage were considered, statistically, to be monoclonal. The resulting hybridomas were routinely grown in the above medium. On occasion, they were grown in a commercial serum-free medium, Hybridoma-SFM™.

2.5.8 Eukaryotic cell transfections

2.5.8.1 Transient transfections: $7.5x10^5$ cells, in 10ml of medium, were plated in 60mm tissue culture dishes, one day prior to transfection. Transfection was carried out using either Tfx-50™ or Tfx-10™ reagent according to the manufacturer's instructions.

2.5.8.2 Stable transfections: Transiently transfected cells were trypsinised, after 48hrs, and subcultured at a split ratio of 1:15 into 60mm tissue culture dishes containing medium supplemented with geneticin. (The concentration of geneticin required, was determined, by splitting a confluent dish of the parental cell line 1:15, into medium containing various concentrations of geneticin. The minimal concentration of geneticin required, to completely inhibit colony outgrowth, was used to select stable transfectants). Medium was replaced, with fresh medium, every four days.

Colonies were picked when they contained approximately 500 cells (see Fig. 4.12). The following procedure was followed. The medium was drained from the dish using a pasteur pipette. Colonies could be seen, by holding the dish at an angle to the light, as opaque patches on the dish surface. Colonies were removed using a P200 tip attached to a Gilson pipetteman. The tip was scraped over the colony while the plunger was slowly released. Gathered cells were expelled into fresh medium (1ml in a well of a 24 well plate) by vigorously pipetting up and down the P200 tip. Before picking any other colonies the dish was washed with 2 volumes of PBS to remove any loosely adherent or floating cells. Colonies were considered to be monoclonal (see Fig. 4.12), and the resulting stable transfectant cell lines were routinely grown in medium supplemented with geneticin.

2.6 Microbiology

2.6.1 Bacterial strains:

E. coli HB2151: *hsdΔ 5thiΔ(lac-proAB)* F'[*traD36 proAB* $^{+}$ *lacI* ^{q} *lacZΔ*M15]

E. coli TG1: *supE hsdΔ 5thiΔ(lac-proAB)* F'[*traD36 proAB* $^{+}$ *lacI* ^{q} *lacZΔ*M15]

E.coli DH5α: *supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

E. coli TOP10F': F'[*lac*I ^{q}*Tn*10(Tet ^{r})] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu)*7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

E. coli XL-1 Blue MRF': Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr)*173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* F'[*pro*AB *lac*I ^{q}ZΔM15 Tn10 (Tet ^{r})]

E. coli XL-1 Blue: *supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^{-}$ F'[*proAB*+ *lacI* ^{q} *lacZΔ*M15 Tn*10(tet* ^{r})]

2.6.2 Culture media

LB Broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl.

SOB (a): 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl.

SOC (a): SOB (a) supplemented with 10mM $MgCl_2$, 20mM glucose.

SOB (b): 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 10mM $MgCl_2$, 10mM $MgSO_4$.

SOC (b): SOB (b) supplemented with 0.4% (w/v) glucose.

2xTY: 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl.

Terrific Broth: 4.7% (w/v) terrific medium, 0.8% (v/v) glycerol.

2.6.3 Bacterial culture

Bacterial strains were maintained as glycerol stocks (bacterial pellets resuspended in 10% (v/v) glycerol in UPH_2O) and stored at -20^{0}C. 5µl of glycerol stock was

streaked on an agar plate (broth supplemented with 1.5% (w/v) agar and antibiotic) and grown overnight at 37^{0}C. The next morning, a colony was picked, and transferred to 10ml of the relevant broth (containing antibiotic) in a 20ml universal container. This culture was grown, during the day, at 37^{0}C in an orbital shaker at 100-200rpm. If necessary, the 10ml culture was used to inoculate 500ml of fresh medium, which was grown for approximately 12 hrs under the same conditions.

2.6.4 anti-NIP ScFv production

A 10ml culture of *E. coli* HB2151 anti-NIP, in 2xTY supplemented with glucose and carbenicillin to final concentrations of 1% (w/v) and 100μg/ml, respectively, was grown at 37^{0}C overnight. The following morning, 500ml of fresh medium was inoculated with 5ml of this culture, and grown at 37^{0}C. The optical density at 600nm of the culture was tested, periodically, and when it reached 0.6, the cells were harvested by centrifugation at 4000g for 20 min. at RT.

The bacterial pellet was washed once in 2xTY, pre-warmed to 30^{0}C, and resuspended in 500ml of 2xTY (again pre-warmed to 30^{0}C) supplemented with carbenicillin to a final concentration of 100μg/ml. 120mg of solid isopropyl β-D-thiogalactopyranoside (IPTG) was added and the culture was grown overnight at 30^{0}C.

The following morning, the culture was cooled on ice for 15 min. and one drop of antifoam A was added to it. The culture supernatant was harvested by centrifugation at 6000g for 10 min. at 4^{0}C. Solid streptomycin was added to the supernatant to a final concentration of 0.15% (w/v) and the supernatant was filtered through a sintered-glass filter and stored at 4^{0}C as a source of ScFv.

A periplasmic lysate was prepared from the cell pellet as a second potential source of ScFv. The pellet was resuspended in 10ml of PBS supplemented with 0.1mM phenyl methyl sulphonyl fluoride (PMSF) and 0.15% (w/v) streptomycin. The pellet was freeze-thawed by storing at -20^{0}C for 1hr. prior to thawing out under a cold tap. This was repeated twice and the supernatant harvested by centrifugation at 6000g for 0.5hr at 4^{0}C. The supernatant was sterile filtered and stored at 4^{0}C.

2.7 Molecular Biology

2.7.1. General Molecular Biological Techniques

As per Sambrook *et al.* (1989) except as described below.

2.7.2 DNA preparations

2.7.2.1 Minipreps: Bacteria were harvested, from a 10ml culture, by centrifugation at 1000g for 10 min. The pellet was 'dried', by centrifugation at 1000g for 30 sec. followed by removal of any traces of supernatant with a pipette. The pellet was resuspended in 200µl of alkaline-lysis solution 1, by vortexing, and was left on ice for 4 min. 400µl of alkaline-lysis solution 2 was added, and they were mixed by rotation and left on ice for 4 min. To this, 300µl of alkaline-lysis 3 solution was added, mixed by vortexing and left on ice for 4 min. 600µl of the resulting mixture was transferred to an eppendorf tube, spun at 12000rpm in a microfuge for 5 min. and the supernatant was transferred to a fresh eppendorf tube.

The supernatant was 'extracted' by adding an equal volume of 50% (v/v) equilibrated phenol/50% (v/v) chloroform (hereafter referred to as phenol-chloroform) to it. The emulsion was mixed by vigorous vortexing and the two layers separated by microfuge centrifugation (hereafter referred to as 'microfuging') at 12000rpm for 2 min. The top layer was transferred to a new eppendorf tube.

An equal volume of isopropanol was added to the eppendorf tube, which was left on ice for 10 min. A dry crude preparation of DNA was recovered, by microfuging at 12000rpm for 5 min. The pellet was rinsed in 1ml of a 70% (v/v) solution of ethanol, left to dry, and resuspended in 50µl of sterile UPH_2O. 1 unit (u) of RNase 1 was added to the solution of DNA in order to destroy any contaminating RNA.

Ultrapure DNA was prepared, if required, from the above preparation, by gel extraction using a Qiaex II gel extraction kit according to the manufacturer's instructions.

*2.7.2.2 **Maxipreps***: Maxipreps of ultrapure DNA were prepared using a Qiagen Maxiprep DNA kit according to the manufacturer's instructions.

*2.7.2.3 **from eukaryotic cells***: Genomic DNA was prepared from eukaryotic cells according to either one of the following two protocols.

i) Cells were harvested from 1 x T-75 flask, and washed in two volumes of PBS. The cells were resuspended in 1ml of UPH_2O and boiled for two minutes. The supernatant was harvested by microfuging at 13000g for 10 min., and was used as a source of genomic DNA.

ii) Cells were harvested from 1 x T-75 flask, and washed in two volumes of PBS. The cells were resuspended in 100µl of PCR lysis buffer, and 1µl of proteinase K was added. The cell suspension was incubated at 65^0C for 10 min. A further 1µl of proteinase K was added and the incubation was continued for a further 50 min. The solution was then incubated at 95^0C for 10 min. The supernatant was harvested by microfuging at 13000g for 10 min. and used as a source of genomic DNA.

2.7.3 Polymerase chain reactions (PCR)

*2.7.3.1 **PCR with Taq polymerase***: The following PCR master mix was prepared: 5.5µl of 40mM stocks of each dNTP, 59µl of 25mM $MgCl_2$, 100µl of 10x *Taq* reaction buffer and 819µl UPH_2O.

PCR reactions were set up as follows: 90µl PCR master mix, 3µl of forward primer (20nmoles), 3µl of reverse primer (20nmoles), 3µl of template DNA ($1x10^6$ molecules) and 0.5µl *Taq* polymerase (1u).

The following temperature profile was used for thermocycling: 94^0C for 1 min. followed by 30 cycles of: 94^0C for 1.5 min., 55^0C for 1 min. and 72^0C for 3 min. Finally, the reaction product was extended at 72^0C for 7 min.

2.7.3.2 PCR with Pwo polymerase: The following PCR master mix was prepared: 5.5µl of 40mM stocks of each dNTP and 478µl of UPH_2O.

PCR reactions were set up as follows: 50µl PCR master mix, 30µl UPH_2O, 10µl of 10x *Pwo* buffer, 3µl of forward primer (20nmoles), 3µl of reverse primer (20nmoles), 3µl of template DNA ($1x10^6$ molecules) and 0.5µl *Pwo* polymerase (1u).

The same temperature profile, as in section 2.7.3.1, was used.

2.7.4 TA cloning and transformation of PCR product

PCR reaction product produced with *Taq* polymerase was directly ligated into the vector pCR™3-Uni provided with the eukaryotic TA cloning kit, according to the manufacturer's instructions. Transformation of the ligation mix into *E. coli* Top10F' chemically-competent cells (provided with the kit) was carried out according to the manufacturer's instructions.

2.7.5 T4 DNA polymerase-mediated-cloning and transformation of PCR product

2.7.5.1 Creation of vector pMIK: The vector pcDNA3.1+ was restricted as follows. 10µl pcDNA3.1+ (1µg), 6µl UPH_2O, 2µl SuRE[1] cut buffer B, 1µl *Hin*dIII (10u) and 1µl *Eco*RI (10u) were mixed incubated at 37^0C for 4 hours.

230µl of UPH_2O was added to the restriction digest, which was extracted with an equal volume of phenol-chloroform. The top layer was removed, and 25µl of 3M sodium acetate and 2µl of a 2% (w/v) glycogen solution were added to it.

500µl of absolute ethanol was added and the mixture incubated at -20^0C for 1 hour. The precipitated, restricted pcDNA3.1+ was pelleted, by microfuging at 13000rpm for 10 min., washed once in 70% (v/v) ethanol, left to air-dry and resuspended in 15µl of UPH_2O.

[1] SuRE cut buffers are a series of reaction buffers, produced by Boehringer Mannheim, and optimised for use with their restriction enzymes. The recipes for these buffers are available in the Boehringer Mannheim catalogue.

The following ligation reaction was set up and incubated at 14^0C overnight. 1µl of restricted pcDNA3.1+ (20fmoles), 1µl of synthetic duplex DNA (see fig. 5.1) (40fmoles), 1µl of 10x ligation buffer, 6µl of UPH_2O and 1µl of T4 DNA polymerase.

The ligation mix was electroporated, into electrocompetent *E. coli* XL1-Blue, as follows. In a cold eppendorf tube, 300µl of the cell suspension was mixed with 2µl of the ligation reaction and left on ice for 30 sec. This mixture was transferred to a cold, 0.2cm electroporation cuvette. The cuvette was placed into the chilled safety chamber slide and pulsed once at the following settings: 25µF, 2.48kVand 200Ω. The cuvette was immediately removed, 3ml of SOC (a) medium added, and quickly mixed, with the contents. The resulting cell suspension, was transferred to a 20ml universal tube, and incubated at 37^0C for 1 hour while shaking at 225rpm.

The transfection mix was plated on LB agar (LB broth with 1.5% (w/v) agar and 100µg/ml carbenicillin) and cultured overnight. 10 colonies were picked and grown in 10ml of LB broth with 100µg/ml carbenicillin. DNA was prepared by miniprep (2.7.2.1.) from half of each culture, and a glycerol stock of the remaining half was also prepared. Each DNA preparation was analysed by restiction digest. A plasmid giving the correct restriction pattern was named pMIK.

2.7.5.2 ***Restriction of pMIK***: Ultrapure pMIK, prepared by maxiprep, was restricted as follows. 40µl pMIK (50µg), 20µl *Eco*RI (200u), 20µl SuRE cut buffer H and 120µl of UPH_2O were mixed in an eppendorf tube and incubated at 37^0C for 4 hours. Subsequently, 200µl of TE buffer was added, and the solution was twice extracted with phenol-chloroform. 400µl of solution was recovered after extraction. To this, 40µl of 3M sodium acetate and 1ml of absolute ethanol was added, and incubated on ice for 30 min. The DNA was pelleted, by microfuging at 13000g for 10 min., washed in 70% (v/v) ethanol, air-dryed and resuspended in 40µl of UPH_2O.

2.7.5.3 pMIK cut back: Two of the following reactions were set up and incubated for 1 hr at 15^0C. 25µl cut back buffer, 5µl T4 DNA polymerase (5u), 5µl of restricted pMIK (6.25µg) and 15µl of UPH_2O. Both reactions were combined and 50µl of TE buffer added to them. The solution was twice extracted with phenol-chloroform and 150µl was recovered. This was extracted with 150µl of chloroform, from which 100µl was recovered. 10µl of 3mM sodium acetate, 1µl 2% (w/v) glycogen and 250µl of absolute ethanol were added and incubated on ice for 15 min. A pellet of DNA was recovered by centrifugation at 13000rpm for 10 min., washed in 70% (v/v) ethanol, air-dried and resuspended in 40µl of UPH_2O.

2.7.5.4 PCR cut back: The PCR product was first purified using a PCR cleanup kit (Boehringer Mannheim) according to the manufacturer's instructions. Two reactions were set up as follows, and incubated at 15^0C for 1 hr. 40µl cut back buffer, 4µl T4 DNA polymerase (4u), 8µl UPH_2O and 8µl purified PCR product (1µg). Both reactions were combined and 70µl of TE buffer added to them. This solution was then treated as per section 2.7.5.3.

2.7.5.5 Ligation: Four of the following ligation reactions were set up, and incubated at 5^0C overnight. 25µl ligation buffer, 5µl cut back vector (200ng), 5µl cut back PCR product (200ng), 5µl UPH_2O, 5µl T4 polynucleotide kinase (50u) and 5µl T4 DNA ligase (5u). The ligation reactions were pooled (180µl) and heated to 65^0C for 15 min. The reaction was phenol-chloroform extracted and 180µl recovered. 18µl of 3M sodium acetate, 1µl of 2% (w/v) glycogen and 450µl of absolute ethanol were added, and incubated on ice for 1 hour. DNA was pelleted by microfuging at 13,000rpm for 15 min., rinsed in 70% ethanol, air-dried for 10 min. and resuspended in 2µl of TE buffer.

2.7.5.6 Transformation: The electroporation chamber and a 0.1cm electroporation cuvette, were pre-chilled on ice. *E. coli* XL1-Blue MRF' electroporation-competent cells were thawed on ice. 960µl of SOC (b) medium was pre-heated to 37^0C.

40µl of the cell suspension was mixed, with 1µl of the purified ligation reaction in an eppendorf tube, on ice. This mixture was transferred to the cuvette, which was placed in the chamber, and pulsed once at 1.7kV, 200Ω and 25µF. 960µl of pre-warmed SOC (b) was immediately added to and mixed with, the contents of the cuvette. The contents were transferred to a 20ml universal tube, and incubated at 37^0C at 200rpm for 1 hour.

A small amount of the cell suspension was then taken to determine the number of transformants present, by plating. The remainder was added to 10ml of terrific broth supplemented with 20µg/ml tetracycline and 50µg/ml carbenicillin, and incubated for 1 hour at 37^0C and 200rpm. 1ml of this culture was added into each of 10 20ml universal tubes containing 10ml terrific broth supplemented with 100µg/ml carbenicillin, and cultured overnight. The next morning the bacteria were harvested from all 10 cultures and the combined bacteria were resuspended in 1ml of a 10% (v/v) solution of glycerol in UPH_2O and this glycerol stock was stored at -20^0C.

2.8 Biochemistry

2.8.1 Solid phase immunoassays

2.8.1.1 ELISAs for titration of antibody activity: 100µl of a solution of antigen in coating buffer (a phosphate buffer, pH 7.6 - see section 2.4.3.1), was added to wells, of a Nunc maxisorp plate, and incubated at room temperature for at least 8 hours.

Wells were washed with 3 x 200µl and 1 x 400µl of wash buffer, and 100µl of a 5% (v/v) solution of FCS in coating buffer was added to each well, and incubated for at least 1 hour at room temperature. This was to 'block' any remaining adsorption sites on the plastic surface.

Wells were then washed out with 4 x 200µl and 1 x 400µl of wash buffer and test samples (100µl per well) were added to the plate. If necessary samples were diluted in PBS containing 0.05% (v/v) Tween 20 and 5% (v/v) FCS (diluent buffer). Samples were incubated for 5 hrs. at room temperature.

Wells were washed with 4 x 200µl and 2 x 400µl of wash buffer, 100µl of secondary antibody (alkaline phosphatase conjugated to a goat anti-mouse antibody or a goat anti-rabbit antibody), diluted as required in diluent buffer, was added to each well and incubated for 2 hours at room temperature.

Wells were washed with 4 x 200µl and 3 x 400µl of wash buffer and 200µl of substrate was added per well. The substrate used was para-nitrophenyl phosphate (pNPP) provided in tablet form and dissolved in the required volume of dH_2O just prior to use. Substrate was left to develop in the dark at room temperature. Optical densities were read at 405nm using a titertek plate reader.

2.8.1.2 ELIspot assay for enumeration of antibody secreting cells: Nunc maxisorp plates were coated and blocked as per section 2.8.1.1.

Wells were washed with 4 x 200µl and 1 x 400µl of wash buffer followed by 4 x 400µl PBS. The population of cells to be analysed, were harvested, washed in three volumes of fresh medium, and resuspended in fresh medium and counted.

Known amounts of cells, in 100µl of fresh medium were added per well, and incubated at 37^0C for 6 hours, in a 5% CO_2 atmosphere.

Wells were washed out with 4 x 200µl and 2 x 400µl of wash buffer, and development with secondary antibody (alkaline phosphatase conjugated to a goat anti-mouse antibody) proceeded as per section 2.8.1.1.

Wells were washed out with 4 x 200µl and 3 x 400µl of wash buffer, and 100µl of substrate was added per well. The substrate used was 5-bromo-4-chloro-3-indolyl phosphate, para-toluidene salt (BCIP) at 1mg/ml in AMP buffer and was prepared fresh. Substrate was left to develop overnight in the dark at room temperature.

The following morning wells were washed with 4 x 200µl and 3 x 400µl of dH_2O and the plate was left to air-dry. The number of blue plaques in each well were counted, under 40x magnification, and the frequency of cells secreting antigen-reactive antibody was calculated, by regression analysis, of the number of plaques per well versus the number of cells per well.

*2.8.1.3 **Affinity analysis using ELISA***: The method of Friguet *et al.*, (1984) was employed. Briefly, the day before the ELISA analysis, a series of antibody-antigen mixtures were prepared in wells of a 96 well plate. These solutions each contained a constant but unknown concentration of antibody. This concentration was nominally referred to as "1". These solutions each contained varying but known concentrations of antigen ([A]). These various antibody-antigen solutions were left overnight at RT, to reach equilibrium.

The following morning, the nominal concentration of free antibody in each solution was determined by ELISA, as per section 2.8.1.1. OD 405nm values were related to nominal concentration values by reference to a standard curve of nominal concentration versus OD 405nm.

The fraction of total antibody, bound by antigen (v), was calculated for each antigen concentration. The slope of a plot of 1/v versus 1/[A] (Klotz plot) gave the dissociation constant for the interaction.

2.8.2 Cytochemistry

2.8.2.1 In situ β-gal staining: Cell monolayers were washed twice with PBS (500µl per well of 24 well plate). 300µl of fix solution was added to each well, for 10 min., and then poured off. This was done twice. 300µl of rinse solution was added to each well and left for 10 minutes before pouring off. This was done twice. 150µl of stain solution was added to each well and left overnight at 37^0C. Cells expressing β-galactosidase were stained blue.

2.8.2.2 In situ c-myc staining: Transiently transfected cells (section 2.5.8.1.) were resuspended at $1x10^6$ cells/ml in culture medium. 50µl volumes were cytospun onto glass slides using the Heraeus labofuge Ae system at 750rpm for 5 min. The resulting cell smears were fixed and permeabilised, using either of the two following procedures.

i) Smears were left air dry for 3-5 min., placed in methanol for 5 min. followed by acetone for 1.75 min., (both solvents at -20^0C). Slide preparations were left dry overnight before use.

ii) Alternatively, smears were left air dry for 3-5 min. before adding a drop of 4% (w/v) paraformaldehyde in PBS to each cell smear. After 10 min. slides were washed for 9 min. with 3 changes of PBS and then placed in a 0.2% (v/v) solution of Triton X-100 in PBS. After 10 min. the slides were washed as before, and were then ready for use.

30µl of affinity-purified (section 2.8.4.2.) anti-c-myc antibody (mouse IgG, produced by clone Myc-9E10), diluted in PBS containing 5% (v/v) FCS, was added to fixed and permeabilised smears, and the slides were incubated in a humid chamber at 4^0C for 1 hour. Slides were washed with 3 changes of PBS for 9 min. and 100µl of a suitable dilution of a FITC-goat anti-mouse IgG conjugate (again in PBS with 5% (v/v) FCS) was added to each smear. Slides were replaced in the

humid chamber at 4^0C for 1 hour. Subsequently, slides were washed for 9 min. with 3 changes of PBS and were left air dry for 5-10 min. Coverslips were mounted on the glass slides with Vectashield mounting medium, and stored in the dark prior to microscopic analysis with a fluorescent Leitz Laborlux K microscope. Cells expressing c-myc fluoresce green.

2.8.3 Immunoblots and related techniques

2.8.3.1 Cell cytoplasm extracts: Cells were harvested from 1 x T-75 flask, washed in two volumes of PBS, resuspended at $5x10^7$ cells/ml in lysis buffer, and incubated on ice for 30-60 min.. The nuclei were removed by microfuging at 250g for 10 min. The supernatant (lysate) was clarified by microfuging at 10000g for 30 min. The lysate was pretreated to remove proteins which may bind bovine serum, mouse IgG or goat IgG. 10µl of bovine serum-agarose, 10µl of mouse IgG-agarose and 10µl of goat IgG -agarose were added per 200µl of lysate, incubated at 0-5^0C for 1 hr., prior to being removed by microfuging at 200g for 5 min. The supernatant was stored at -20^0C.

2.8.3.2 Organ extracts: Extracts were prepared from the following Balb/C organs: heart, liver, lung and spleen. The relevant organ was removed, from the required number of mice, and roughly minced with a scalpel. Approximately 600mg of the minced organ was added to 10ml of homogenising buffer, in a 20ml universal tube, and was disrupted with a homogeniser for 4 minutes. The resulting mixture was clarified by centrifugation at 6000g for 10 minutes. The supernatant was aliquotted and stored at -20^0C. The protein concentration was determined spectrophotometrically, using the following formula (Peterson, 1983):

$$\text{conc. (mg/ml)} = \text{OD 205nm} / [27 + 120(\text{OD 280nm}/\text{OD 205nm})]$$

*2.8.3.3 **SDS-PAGE***: A 5% (w/v) stacking/10% (w/v) resolving SDS-PAGE gel was prepared using an Atto dual minislab electrophoresis system. Resolving gel: 1.98ml solution A, 1.5ml solution B, 2.52ml dH_2O, 50µl 0.5% (w/v) ammonium persulfate and 5µl TEMED. Stacking gel: 0.33ml solution A, 0.5ml solution C, 1.15ml dH_2O, 30µl 0.5% (w/v) ammonium persulfate and 5µl TEMED. The gel was poured according to the manufacturer's instructions with the following exception. Wells, in general, were created in the stacking gel by insertion of a comb in the unpolymerised gel. For running organ extracts through the gel, however, a level interface was created by pouring ethanol on top of the unpolymerised stacking gel. After polymerisation, the ethanol was poured off to leave a level interface.

600µg of the relevant organ extract (in a volume of less than 100µl) was mixed with ¼ volume of sample buffer, boiled for two minutes and loaded along the entire length of the top of the gel. Other protein samples were mixed with ¼ volume of sample buffer, boiled for two min. and 10µl was loaded into individual wells. The samples were run through the stacking gel at an ampage of 15mA and through the resolving gel at an ampage of 20mA.

SDS-PAGE gels were either stained directly, or transferred to nitrocellulose (NC) (see section 2.8.3.4). Gels were stained by incubating in Coomassie Blue stain solution at RT for 1 hr. on a rocker platform. The gel was then destained by incubating at RT for a couple of days in multiple changes of destain solution, while constantly rocking on a rocker platform. Protein bands stain blue against a clear background.

*2.8.3.4 **Electrotransfer***: The Bio-rad Trans-blot cell was used for electrotransfer of proteins, from the SDS-PAGE gel, to nitrocellulose membranes. The SDS-PAGE gel was removed from between the glass plates and incubated for five minutes, with rocking, in five changes of transfer buffer. Electrotransfer was then carried out overnight, at 30V and 100mA, according to the manufacturer's instructions.

2.8.3.5 Immunoblotting: The nitrocellulose was removed from the Trans-blot cell, and was blocked for 0.5 hours with PBS containing 0.3% (v/v) NP40 followed by PBS with 0.1% (v/v) Tween 20, for 2 hrs, while shaking on a rocker platform.

The blocked nitrocellulose was, in general, probed with primary antibody (mouse antibody diluted as required in PBS containing 0.05% (v/v) Tween-20) for 2 hrs at RT with constant rocking. Organ extracts, transferred to NC, were probed with the aid of a miniblotter. This is a multichannel instrument, which allowed incubation, in parallel, and simultaneously, of many probes with the one nitrocellulose membrane, while minimising the amount of probe required. Primary antibodies were incubated with the blot for 4 hours at RT, while the miniblotter was rocked at 6 tilts per minute.

The blot was, subsequently, washed for a half-hour with 6 changes of PBS with 0.05% (v/v) Tween 20, while rocking constantly. The blot was then probed with a secondary (alkaline phosphatase conjugated to goat anti-mouse antibody) antibody, diluted in PBS with 0.05% (v/v) Tween 20 and 5% (v/v) FCS. Incubation was for 2 hours at RT with constant rocking.

The blot was then washed for half an hour, with 6 changes of PBS with 0.05% (v/v) Tween 20, prior to addition of substrate to the blot. Substrate was 0.2mg/ml BCIP and 0.2mg/ml NBT (nitroblue tetrazolium) dissolved in AMP buffer. Substrate was incubated with the blot at RT, until sufficient colour had developed, and the reaction was then terminated, by washing the blot with water.

2.8.4. Protein G affinity purification of IgGs

*2.8.4.1 **Concentration of tissue culture supernatant***: 200ml of spent supernatant from the elevant hybridoma cell line was collected over a period of time, supplemented with sodium azide to a final concentration of 0.02% (w/v) and stored at 4^0C.

The supernatant was ultrafiltered to a final volume of 20ml, using a stirred ultrafiltration cell with a 76mm diaflo ultrafilter membrane with a molecular weight cut-off of 100,000 daltons. The concentrate was stored at 4^0C.

*2.8.4.2 **Affinity purification***: 1ml of a suspension of immobilised protein G (immobilised on Sepharose 4B) was packed in a chromatography column. The column (stored in PBS containing 20% (v/v) ethanol) was pre-equilibrated with 20ml of PBS.

Either, 1ml of serum or 20ml of concentrated supernatant was passed through the column. The flow-through was collected and allowed to flow through the column a second time.

25ml of PBS was passed through the column and, subsequently, the retained protein was eluted with 0.1M glycine buffer (pH 2.7). 0.9ml fractions of eluate were collected in eppendorf tubes, and neutralised with 0.1ml of 1M Tris.Cl buffer (pH 8.7.).

The optical density of each fraction, at 280nm, was measured. Those fractions containing significant protein were pooled, dialysed against PBS with 0.5M NaCl, and stored at 4^0C.

2.8.5 Generation of affinity columns

Cyanogen bromide (CNBr)-activated Sepharose 4B was swollen in 0.1M HCl for 1 hr. It was sucked dry on a Buchner funnel, and washed (on the Buchner funnel) twice with 250ml of PBS. 6g (dry-weight) of the gel can be expected to give 20ml of wet gel.

The ligand was dissolved in coupling buffer (0.1M carbonate buffer (pH 8.3) containing 0.5M NaCl), in general, at 1-10mg/ml. A small volume was kept for later analysis. Equal volumes of ligand solution and wet gel were mixed, and incubated overnight at 4^0C, with constant rotation.

The gel was let fall out of suspension, and a small sample of solution was taken. Spectrophotometric comparison of this solution with the solution taken prior to incubation , allowed one to calculate, roughly how much of the ligand had bound to the Sepharose.

The gel was washed sequentially (on a Buchner funnel), with 200mls of 0.1M carbonate buffer (pH 8.5), PBS and PBS containing 1M NaCl.

The gel was incubated with 1M ethanolamine (pH 9.0), for 2 hrs. at RT, with constant rotation.

The gel was washed sequentially with 200mls of 0.1M NaAcetate buffer (pH 4.0), 0.5M NaCl and coupling buffer. The affinity column was stored in PBS with 0.02% (w/v) NaN_3 at 4^0C.

2.8.6 Affinity purification of mouse IgM from hybridoma supernatants

A commercial preparation of affinity-purified anti-mouse IgM (developed in sheep), was coupled to CNBr-activated Sepharose 4B, for the purposes of purifying mouse IgM. The basic protocol of section 2.8.5 was used, and exact details are given in section 3.3.2.2. The final 10mls of wet gel were packed into a chromatography column and the final IgM purification scheme, chosen, is described below.

The column was equilibrated, by passing 60ml of PBS through it. 10ml of concentrated hybridoma supernatant (see section 2.8.4.1) was passed through the column, followed by 60ml of PBS. Protein retained on the column were eluted by slowly passing 30ml of 0.1M glycine buffer (pH 2.2) through it and collecting 1ml fractions. These fractions were neutralised by adding 100µl of 1M Tris.Cl (pH 8.7). 60ml of elution buffer was then quickly passed through the column to remove any remaining IgM. 100ml of PBS was then finally passed through the column, which was stored in PBS + 0.02% NaN_3, at 4^0C.

The optical density of each fraction was determined spectrophotometrically at 280nm, and fractions containing significant amounts of protein were pooled, dialysed against PBS with 0.5M NaCl, and stored at 4^0C.

2.8.7 'Real-time' Biomolecular Interaction Analysis

The Biacore™ with CM5 chips, was used for all real-time analyses. For a detailed description of the theory and practice of this instrument, the reader is referred to Jonnson *et al.*, (1991). All the experiments, to be described herein, relied on NHS/EDC coupling chemistry, to immobilise ligand on the dextran matrix at the surface of the chip.

The ligand was dissolved at between 5-50μg/ml in 0.01M NaAcetate buffer, at a range of pH's between 4-5. These were passed, sequentially, over an underivatised chip surface, and that pH giving the greatest mass (measured in terms of response units (RU)) pre-concentrated at the surface of the chip, was used for ligand immobilisation.

The chip surface was activated, by passing 35μl of a solution containing 0.05M NHS and 0.2M EDC in UPH_2O, over the chip surface at a flow rate of 5μl/min. 35μl of a solution of the ligand, in the chosen buffer, was passed over the surface at a flow rate of 5μl/min. Remaining active sites were deactivated, by passing 35μl of a 1M ethanolamine (pH 8.5) solution over the surface at a flow rate of 5μl/min. The surface was then used, to analyse biomolecular interactions involving that particular ligand.

2.9 Animal experimentation

2.9.1 Mice

All Balb/C mice used in these studies were housed in the DCU animal house and cared for by a qualified vet. All C57BL\10 mice were purchased, freshly killed, from the UCD Biomedical Facility, Dublin.

2.9.2 Rabbits

One NZW rabbit was used during the course of these studies for immunisation purposes. The rabbit was purchased from the Trinity Bioresources Unit, Dublin, and cared for by a qualified vet in the DCU animal house where all experimentation took place. These studies were carried out under licence from the Department of Health. The immunisation protocol used is reproduced here

Day 1: Immunise rabbit by intradermal injection with an emulsion (1ml) consisting of a 200μg/ml solution of the immunogen mixed 1:1 with Freunds Complete Adjuvant.

Day 21: Remove a 1ml blood sample from the ear vein (primary bleed) and titrate its anti-immunogen activity.

Day 31: Re-immunise the rabbit as before using Freunds Incomplete Adjuvant.

Day 41: Obtain blood sample as before (secondary bleed) and titrate as before.

Antiserum recovery:

The above cycle of blood sampling/titration and re-immunisation is carried out at 10 day intervals until the activity is seen to plateau. At this stage the animal is sacrificed and the blood recovered by cardiac puncture.

CHAPTER 3

IN VITRO IMMUNISATION AND ANTIBODY POLYREACTIVITY

One of the main areas of interest in the laboratory has, and continues to be, the use of alternative methods, to *in vivo* immunisation, for the production of monoclonal antibodies. Antibody-library technologies have been exploited to produce, for example, anti-coumarin ScFv antibodies (Killard *et al.*, 1997). Another method, frequently employed, is *in vitro* immunisation (IVI). Nonetheless, at the outset of this project, a review of the literature (discussed in section 1.3.2.2) suggested, not only that many of the supposed advantages of this technique, over *in vivo* immunisation, were more apparent than real, but that a solid case could be made in favour of the hypothesis that Mabs produced in this fashion may suffer from polyreactivity.

This hypothesis was predicated on one fact, re: IVIs, in general, only result in primary immune responses. It was also based on the idea, that B1a cells may be preferentially activated during IVI. This notion was justified by a review of the literature. i) Direct evidence has been published, that an IVI culture system did result in preferential activation of CD5+ B cells (Feeney *et al.*, 1984). ii) A number of reports have indicated that no requirement exists *in vitro* for either antigen-specific T cells (Feeney *et al.*, 1984) or, indeed, any T cells (Borrebaeck and Moller, 1986). IVI responses to unconjugated haptens have even been reported, *in vitro* (Stahl *et al.*, 1995; Bonwick *et al.*, 1997). These observations dovetail nicely with the finding, that murine B1a cells but not B2 cells, proliferate and secrete antibody in response to IL-5, in a T cell independent fashion (Kasaian and Casali, 1993). iii) finally, CD5+ B cells appear hardier than conventional B cells, *in vitro*, and survive longer (Pritsch *et al.*, 1997).

In order to test the hypothesis, a number of splenocyte culture systems were initially investigated to ascertain, whether they supported a humoral immune response (section 3.1). One culture system, which supported an immune response was used to produce a panel of 9 hybridomas (section 3.2). Finally, a series of studies aimed at analysing the binding-characteristics of their respective Mabs were carried out (section 3.3). These studies, in their entirety, allow a number of conclusions to be drawn. In particular, the evidence is strongly in favour of the notion, that IVI tends to produce polyreactive Mabs.

3.1 *IN VITRO* IMMUNISATIONS

As mentioned in section 1.3.2.2, the reproducibility of IVIs was questioned. Therefore, two IVI systems were investigated to determine whether they actually supported an antigen-reactive, antigen-induced, humoral immune response, this being defined as a variation in the frequency of cells secreting antibody against the antigen, upon exposure to it. Initial studies (section 3.1.2) focussed on an IVI system for murine splenocytes, originally published by Ossendorp *et al.* (1986). This was chosen as it had been successfully adapted and used in the laboratory previously (Carroll *et al.*, 1991). The second, a system for murine splenocytes originally published by Borrebaeck and Moller (1986), was chosen at random. Studies of this system are reported in section 3.1.3. First, the development of an ELIspot assay, in order to facilitate the analysis of these two IVI culture systems, is reported (section 3.1.1).

3.1.1 ELIspot assays: ELIspot assays (reviewed by Sedgewick and Holt, 1986; see also Fig. 3.1) allow enumeration of the frequency of cells secreting antibody reactive with a given antigen and, hence, allow evaluation of humoral immune responses at the cellular level.

In order to develop and validate an ELIspot assay, an IVI was set up as described in section 2.5.6.1, with no added antigen, and cultured for 42 hrs. The frequency of cells in this population, secreting anti-dinitrophenol-human albumin[1] (DNP) was evaluated by an ELIspot assay, essentially as described in section 2.8.1.2. DNP was used as the coating antigen at 100, 10 and 0μg/ml. Alkaline phosphatase, conjugated to goat anti-mouse polyvalent immunoglobulin, was used as the secondary antibody, at some or all of the following dilutions: 1:400, 1:800, 1:1600, 1:3200, 1:6400 and, also, with no secondary antibody (referred to as a dilution of '1:infinity'). The results are depicted in Figs. 3.2-3.5.

[1] Throughout this chapter, anti-antigen refers to antibody against that antigen.

Fig. 3.1: *ELIspot assays allow the enumeration of the number of cells secreting antibody against an antigen of interest. In order to carry out an ELIspot assay, the wells of a 96-well plate are coated with the antigen of interest (A) and then blocked (B). The cells to be tested are washed and added to the well, where they fall out of suspension and form a monolayer on the coated surface of the well (C). If any cell is secreting antibody, reactive with the coating antigen,* ***it is bound locally in the vicinity of the cell*** *(C). The bound antibody is visualised, subsequently, in a two-step process. Firstly, the primary antibody is bound by a secondary antibody (an antibody against the primary antibody, conjugated to alkaline phosphatase) (D and E). Finally, a solution of 5-bromo-4-chloro-3-indolyl phosphate, p-toluidene salt (BCIP), a substrate for alkaline phosphatase is added to the wells. When acted upon by the enzyme, BCIP is converted to an insoluble product. This insoluble product precipitates out* ***locally in the vicinity of the cell*** *to form what is termed a 'plaque' (F). Many blue plaques are shown in the photograph (G). Each plaque is assumed to be the result of one cell, only, secreting antibody against the coating antigen. The number of plaques per well corresponds to the number of cells per well secreting antibody against the coating antigen. A plot of the number of plaques per well versus the number of cells per well should, as a result, give a straight line through the origin. The slope of the resulting 'best-fit-line' indicates the fraction of the cell population secreting antibody against the coating antigen.*

Figs. 3.2-3.5 continued: *DNP-human albumin (100, 10 and 0μg/ml) and dilution of secondary antibody. Frequencies are measured as antibody-secreting cells (ASC) per $1x10^6$ splenocytes. The values for ASC/$1x10^6$ splenocytes were determined from a linear regression analysis of all the trend lines, of plaques versus cells, depicted in Figs. 3.2-3.4. Such a regression analysis gives one the mean ± the standard deviation of the slope of the 'best-fit-line' to the trend. The depicted values for ASC/$1x10^6$ splenocytes are then calculated by multiplying the mean ± the standard deviation by $1x10^6$.*

These 'ASC/$1x10^6$ splenocytes' values were considered a valid, albeit imperfect measure of the frequency of cells secreting anti-DNP antibody. Quite clearly, as plaque numbers were linearly related to cell numbers, and as plaque formation was completely dependent upon the use of secondary antibody (see Figs. 3.2-3.4), plaque formation was causally linked to the secretion of antibody by cells. In this sense, the assay was valid.

However, although all plaques were due to the secretion of antibody by cells, it was equally clear, that not all plaques could be attributed to cells secreting anti-DNP antibody. In wells, which were blocked only, significant numbers of plaques developed (Fig. 3.4). This was not surprising and was attributed to cells secreting antibody against the blocking agent (FCS) or, perhaps, the plastic. The number of plaques developed in uncoated, blocked wells could not be significantly reduced by suspending the test cells in medium spiked with 10% (v/v) FCS. Given that wells coated with DNP may also contain adsorbed FCS and, by definition, contain a plastic solid phase, some of the plaques developed in these wells may not be attributable to cells secreting anti-DNP. Unfortunately, there was no way of determining the fraction of the plaques developed in DNP-coated wells, which were due to cells secreting antibody against DNP, and in this sense, the assay was imperfect. Hence, the 'ASC/$1x10^6$ splenocytes' values were referred to as the 'apparent frequency' of cells secreting antibody against DNP.

The above considerations indicated that a 100μg/ml solution of coating antigen was optimal. Not only did this coating concentration give the greatest apparent frequency of cells secreting anti-DNP; it would be expected to reduce the amount of FCS adsorbed onto the plastic surface.

These results (Fig. 3.5) clearly demonstrated that a coating concentration of 100μg/ml DNP-human albumin, (DNP), gave the greatest sensitivity, i.e. the greatest apparent frequency of cells secreting anti-DNP. Subsequent ELIspots always used coating antigen at this concentration, unless explicitly stated otherwise.

While, ostensibly, these results (Fig. 3.5) appeared to suggest that higher concentrations of the secondary antibody may be useful, this was not so. As presented, these results give no indication of the problem of artifactual ('background') colour development, which hindered counting of plaques. A visual examination of the developed wells resulted in a 1:3200 dilution of the secondary antibody being chosen as optimal and was used in all subsequent ELIspot experiments.

3.1.2 Evaluation of the method of Ossendorp *et al.* (1986): To determine, whether the method of Ossendorp *et al.* (1986) facilitated an antigen-reactive, antigen-induced, immune response, the effect of the concentration of GIgG in the splenocyte culture, on the frequency of cells secreting anti-GIgG, was investigated. This was investigated directly, at the single-B cell level, using the ELIspot assay (section 3.1.2.1). It was also analysed indirectly, by immortalising the cultured splenocytes, as hybridomas, and screening the resulting hybridoma populations for anti-GIgG secretion (section 3.1.2.2).

GIgG was chosen as the antigen after an ELIspot analysis of the apparent frequency of naturally activated splenic B cells (see section 1.2.2) secreting antibody against a variety of antigens. Antigens used were DNP-human albumin (DNP), digoxigen-keyhole limpet haemocyanin (KLH), 7-hydroxycoumarin-ovalbumin (7-OHC), porcine thyroglobulin (Thyr), goat IgG (GIgG) and lipopolysaccharide from *E. coli* 055:B5 (LPS). An antigen, which in this thesis is referred to as M6149, was also used. M6149 refers to the IgG fraction, purified by protein G affinity chromatography (section 2.4.8.2), of an antiserum reactive with mouse polyvalent immunoglobulins. This antigen could act as a capture antigen for any mouse antibody, regardless of its subclass or idiotype, and was used to determine the frequency of cells secreting antibody.

The results from this experiment are depicted in Fig. 3.6. The natural response to GIgG was very low (the apparent frequency of cells secreting anti-GIgG was only 20 ASC/1x10^6 splenocytes, at a coating concentration of 100µg/ml of GIgG). Because of this low natural response to GIgG, it was decided to use this antigen as a model antigen in all studies.

3.1.2.1 **Direct analysis:** A series of IVIs were set up as described in section 2.5.6.1. Each individual culture received a different concentration of GIgG (0, 0.01, 0.1, 1 or 10μg/ml). After 72 hrs., the apparent frequency of cells secreting any antibody, antibody against GIgG or antibody against DNP was determined for each culture. These apparent frequencies are plotted in Fig. 3.7 as a function of the concentration of GIgG.

If an antigen-reactive, antigen-induced immune response occurred in the culture system, the anti-GIgG cellular response would be expected to vary with the concentration of GIgG in the culture. The anti-DNP response and the total number of cells secreting antibody would be expected to be invariant with changing GIgG concentration. Analysis of variance of the data presented in Fig. 3.7 (Ostle and Malone, 1988) using a 95% confidence limit indicated, that the apparent frequency of cells secreting anti-GIgG was independent of the concentration of GIgG in the culture. In other words, there was no evidence to suggest that an antigen-reactive, antigen-induced, immune response was occurring in this culture system. Surprisingly analysis of variance indicated that, the apparent frequency of cells secreting anti-DNP did depend upon the concentration of GIgG used. This was most likely due to the response at a GIgG concentration of 0.01μg/ml. This response may be an outlier and no practical significance was attached to this statistical finding. In subsequent experiments, this finding could not be repeated. The apparent frequency of antibody-secreting cells was, as expected, invariant with GIgG concentration.

While no specific immune response was obtained, a polyclonal immune response obviously occurred in this culture system. Under microscopic analysis colonies of proliferating cells were evident (Fig. 3.8). Also, the frequency of antibody-secreting cells was higher after the 72 hr. culture period, than it was prior to culture (approximately 8000 ASC/1x10^6 splenocytes, versus 3180 ASC/1x10^6 splenocytes prior to culture).

Fig. 3.6 **Fig. 3.7**

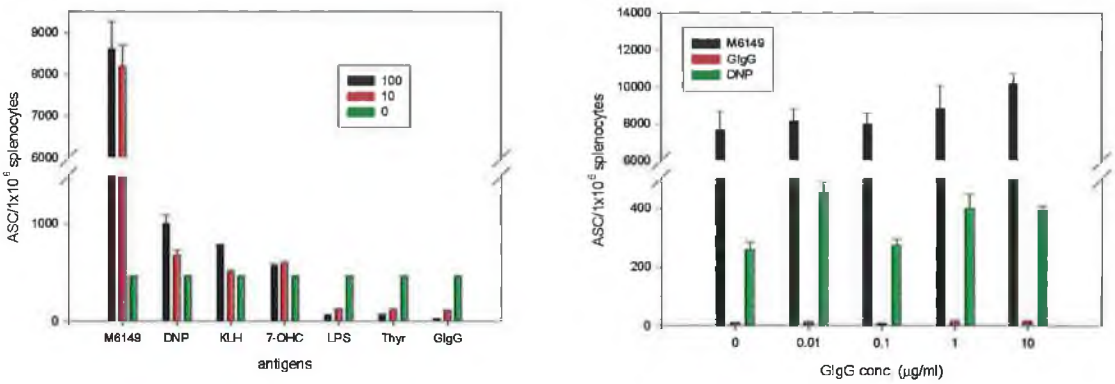


Fig. 3.6: *Splenocytes were obtained from unimmunised Balb/C mice as described in section 2.5.6.1 and the apparent frequency of cells secreting antibody, reactive with a number of different antigens, was determined by ELIspot assay. It was evident that, the natural immune response varied greatly against different antigens. The natural immune response against GIgG appeared very low (20 ± 2 ASC/1x10^6 splenocytes, at a coating concentration of 100µg/ml).*

ELIspot: The ELIspot assay was carried out as described in section 2.8.1.2. Coating antigens were used at a concentration of 100 (black), 10 (red) or 0µg/ml (green). DNP: DNP-human albumin; KLH: digoxigen-keyhole limpet haemocyanin; 7-OHC: 7-OHC-ovalbumin; Thyr: porcine thyroglobulin; GIgG: goat immunoglobulin G; LPS: lipopolysaccharide from E. coli 055:B5. An alkaline phosphatase, conjugated to goat anti-mouse polyvalent immunoglobulin, was used as the secondary antibody at a dilution of 1:3200

Fig. 3.7: *IVIs were set up, as described in section 2.5.6.1, using different amounts of GIgG as antigen, and cultured for 72 hrs. The apparent frequencies of immunised splenocytes secreting anti-GIgG, anti-DNP or antibody, was determined by an ELIspot assay and plotted as a function of GIgG conc. No evidence was obtained to suggest that an immune response had occurred against GIgG*

ELIspot assays:These were carried out as described in section 2.8.1.2. Either GIgG (anti-GIgG), DNP (anti-DNP) or M6149 (antibody), at 100µg/ml were used as the coating antigen. An alkaline phosphatase, conjugated to goat anti-mouse polyvalent immunoglobulins, was used as the secondary antibody, at a dilution of 1:3200 .

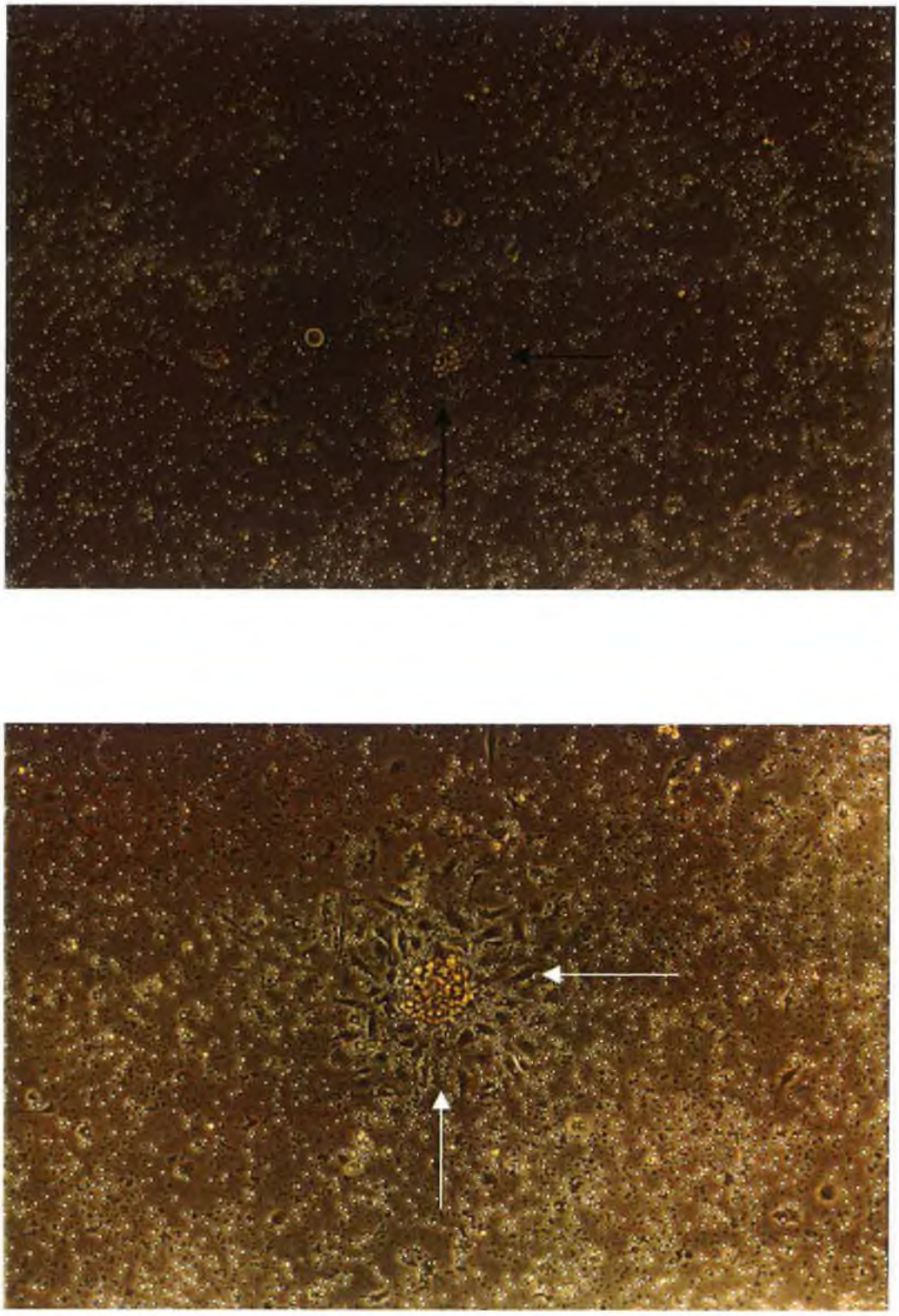


Fig. 3.8: *Colonies of proliferating cells are clearly visible in the above two photographs (40x magnification). A small colony of proliferating cells is visible in the top photograph. A much larger colony, with an associated adherent cell (presumably macrophage) colony, is visible in the bottom photograph.*

3.1.2.2 **Fusion analyses:** To corroborate the results reported in section 3.1.2.1, which suggested that no antigen-reactive, antigen-induced humoral immune response was occurring in culture, IVIs were set up as described in section 2.5.6.1, utilising GIgG as antigen at 0, 0.01, 0.1, 1 and 10μg/ml. After 72 hrs, individual cultures were fused as described in section 2.5.7.1. Poisson distribution analysis (see De Blas *et al.*, 1981) of fusion masterplates, on day 7 after fusion, indicated that the hybridomas were outgrowing at statistical monoclonality. Thus, individual wells were treated, and referred to, as clones. Clones were screened by ELIspot assay, as described in section 2.5.7.2, for both antibody secretion and anti-GIgG activity. (The reasons for choosing this screening system and its validation are discussed below). The decision to classify a clone as positive or negative was made as follows. If a clone gave absolutely no plaques, it was to be considered negative. Even the presence of one plaque was taken as indicating that the clone was positive. Fig. 3.9 shows the percentage of antibody-secreting clones, which secreted anti-GIgG, as a function of GIgG concentration. This percentage was invariant (0%) with GIgG concentration and, hence, this analysis clearly indicated that no antigen-dependent, antigen-reactive, immune response occurred in culture.

The decision to utilise an ELIspot screen rather than the more conventional ELISA screen was made for two reasons. Firstly, as the humoral immune response was being directly investigated by screening B cells for antibody secretion at the single-B cell level, it seemed sensible and consistent to screen hybridoma clones in the same fashion. Secondly, Spira and Sharff (1992) reported that an ELIspot assay was less prone to false positives than an ELISA, in screening for hybridoma subclones, which had undergone an isotype switch.

This screen was validated as follows. Firstly, as masterwells contain dying splenocytes as well as hybridoma cells, it was necessary to demonstrate that only hybridoma cells could generate plaques. This was demonstrated by showing that mock fusions (fusion in the absence of Sp2/0s) did not result in any plaque formation (results not shown). Thus, plaque formation was due to antibody secretion by hybridomas and not splenocytes. Secondly, although the decision to classify a clone as positive or negative based on the presence or

absence of only one plaque may suggest that this particular screen should be susceptible to many false positives and negatives due to random error, this was not so. In practice, clones subsequently classified as positive always gave rise to significant numbers of plaques (>10), and clones subsequently classified as negative always gave rise to no plaques. Thus, a clear discrimination between positive and negative wells was achieved with this assay and it was considered very unlikely that, random error generated false positive or negatives. Finally, although the possibility of false negatives due to systemic error was not considered, the possibility of false positives due to systemic error was. Evidence, presented more fully in section 3.2, suggests that there was no significant problem with false positives. Of 10 clones identified, using the ELIspot screen, as positive for anti-GIgG secretion, 9 were shown using an independent ELISA-based assay, to have significant affinity for GIgG.

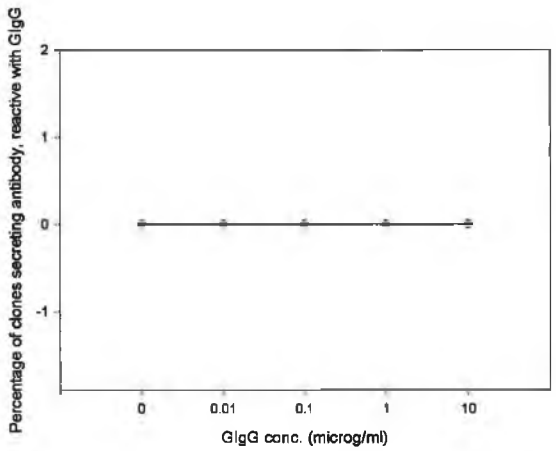
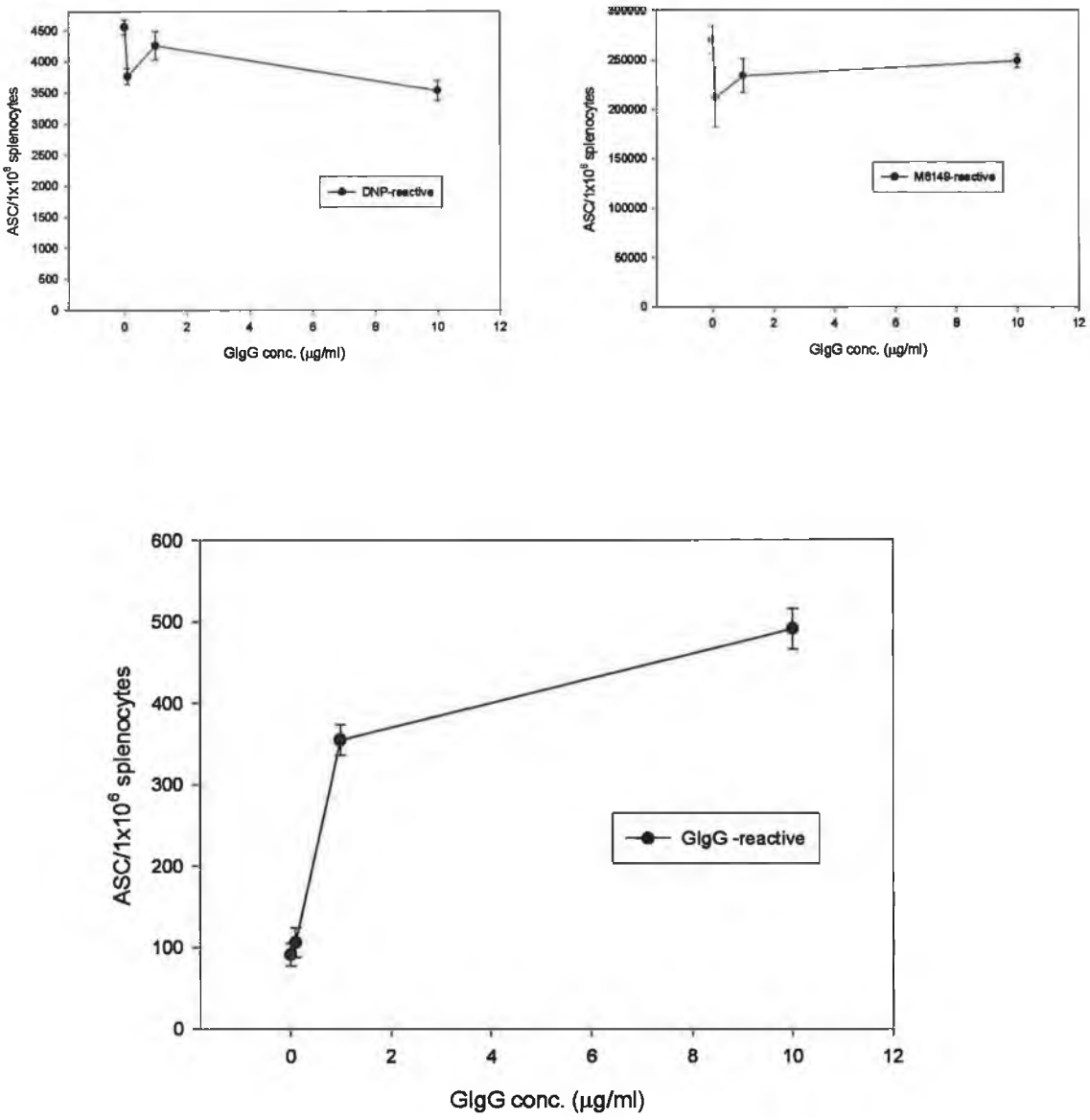


Fig. 3.9: *IVIs were set up as described in the legend to Fig. 3.7, fused according to section 2.5.7, and the resulting clones were analysed by ELIspot assay, for both antibody secretion, and anti-GIgG activity. The percentage of antibody-secreting clones, secreting anti-GIgG was calculated, in each case, from analyses of at least 33 antibody-secreting clones.*

ELIspot: the ELIspot assay was carried out essentially as described in section 2.5.7.2. Either GIgG (anti-GIgG) or M6149 (antibody secretion) were used as coating antigens at 100μg/ml. An alkaline phosphatase, conjugated to goat anti-mouse polyvalent immunoglobulin was used as secondary antibody, at a dilution of 1:3200. A well was considered positive for either anti-GIgG secretion or antibody secretion, if one or more plaques developed.

3.1.3 Evaluation of the method of Borrebaeck and Moller (1986): The negative results presented in section 3.1.2 indicated that it was necessary to try another variant of the IVI technique, and that published in the above reference was chosen. A series of splenocyte cultures were set up as described in section 2.5.6.2. Each culture received a different amount of GIgG (0, 0.1, 1 or 10μg/ml) as an antigen and was cultured for a period of 5 days. At the end of this period, the apparent frequency of cells secreting anti-GIgG, anti-DNP or antibody was determined for each culture, by ELIspot assay. The results are depicted in Fig. 3.10.

A significant increase in the apparent frequency of cells secreting anti-GIgG was evident with increasing concentration of GIgG. Quite clearly, this culture system did allow an antigen-dependent, antigen-reactive, immune response. Of note, this response was superimposed on a large polyclonal immune response. This is indicated by the very large apparent frequency of antibody-secreting cells on day 5, for all GIgG concentrations (≈ 220,000 ASC/1x10^6 splenocytes compared to ≈ 2,500 ASC/1x10^6 splenocytes, prior to culture). Indeed, significantly greater proliferation occurred in this culture system (Fig. 3.11) than in the previous system (Fig. 3.8).



Figs. 3.10: *IVIs were set up as per section 2.5.6.2. GIgG was used as antigen at concs. of 0, 0.1, 1 and 10µg/ml. The splenocytes were cultured for 5 days prior to analysis, by ELIspot assay, of the cellular response to M6149, DNP and GIgG. The results are plotted as a function of GIgG conc. The cellular response to GIgG was obviously dependent upon the concentration of GIgG added to the culture; a clear humoral immune response had occurred in this culture system.*

ELIspot: The ELIspot assay was carried out as described in the legend to Fig. 3.7.

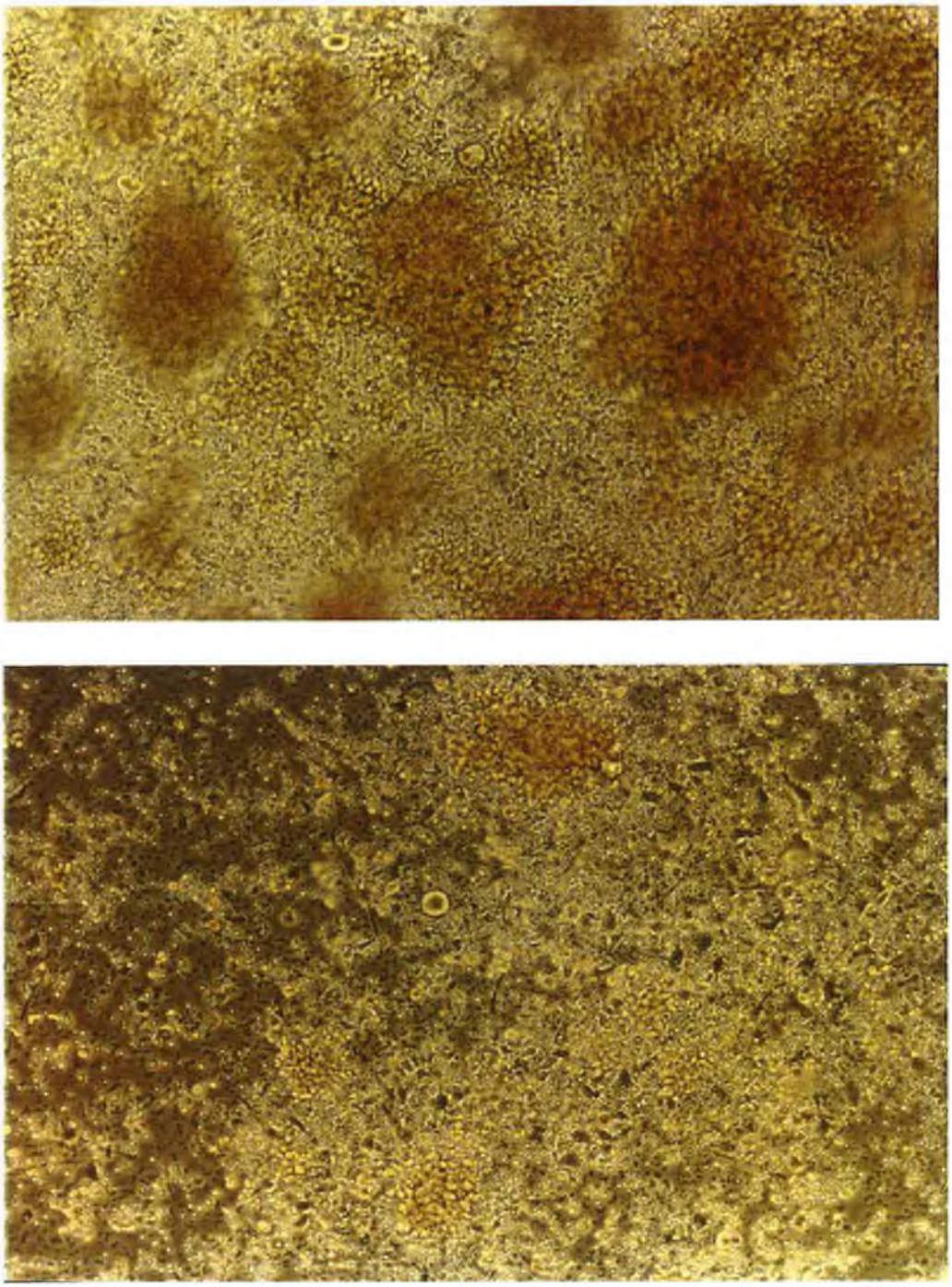


Fig. 3.11: *Two photographs (40x magnification) are presented of splenocytes cultured for 5 days as described by Borrebaeck and Moller (1986). A great degree of cell proliferation was evident in this culture system.*

3.2 Production of a panel of Mabs reactive with Goat IgG

Having established that the splenocyte culture system of Borrebaeck and Moller (1986) facilitated an immune response, it was used to produce a panel of hybridomas secreting anti-GIgG. An IVI was set up as per section 2.5.6.2 using GIgG as antigen at 1μg/ml. After a culture period of 5 days, the cells were fused as described in section 2.5.7.1. Masterwells were screened on day 9 after fusion, as described in section 3.1.2.2, for both antibody secretion and anti-GIgG activity. Of 219 masterwells secreting antibody, 39 (18%) showed anti-GIgG activity. (An IVI was also set up without any GIgG and subsequently was treated as the above IVI was. In this instance, of 101 wells in which antibody secretion was demonstrable, only 1 (1%) showed any anti-GIgG activity. Thus, as expected, based on the results presented in section 3.1.3.1, the presence of GIgG in the IVI increased the frequency of hybridomas secreting anti-GIgG).

Of the 39 positive wells, 13 hybridomas in total were cloned as described in section 2.5.7.3. These were named G1, G3, G4, G7, G9, G11, G18, G19, G22, G24, G30, G31 and G33. G7, G9 and G22 proved unstable and were not used in any analyses. G31 proved unreactive with GIgG when the affinity of their interaction was measured (see section 3.3.1). The reason for this false positive is unknown but probably may be attributed to reactivity with the blocking agent used during the screening assay. The remaining 9 hybridomas were subsequently characterised.

3.2.1 Titration and isotyping of Mabs: Supernatants were harvested from all 9 hybridomas. Both the quantity of Mab and the anti-GIgG activity possessed by each supernatant, were titrated by ELISA (Figs. 3.12-3.22). These ELISAs served two functions. Firstly, they allowed a crude comparison of the anti-GIgG activity of each supernatant. Secondly they allowed the isotyping of the Mabs heavy chains.

The ELISA results obtained with a G1 supernatant are depicted in Figs. 3.12-3.14. Surprisingly this Mab appeared to be of both μ and γ isotypes (see Figs. 3.12 and 3.13). Further experimentation (results not shown) indicated, however, that the anti-γ conjugate was just as reactive with mouse IgM as it

Fig. 3.12

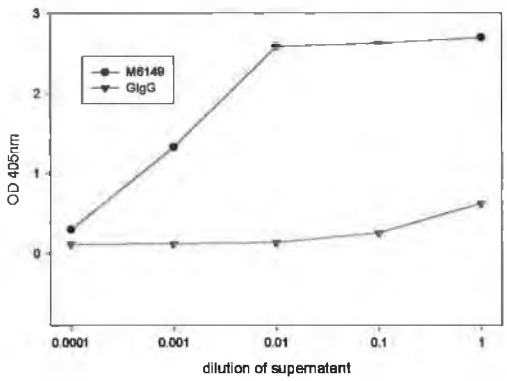


Fig. 3.13

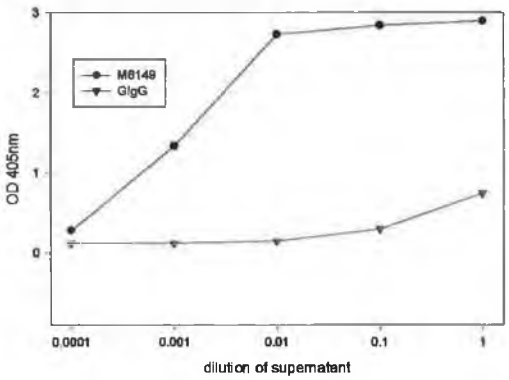
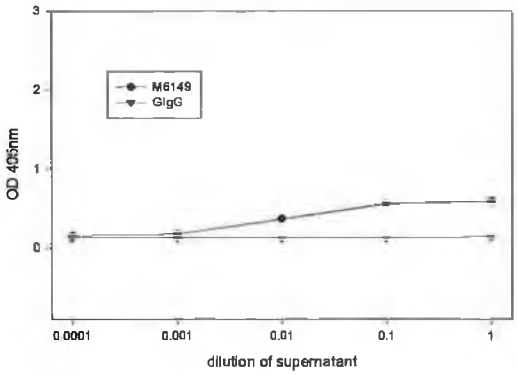


Fig. 3.14



Figs 3.12-3.14: *The quantity of Mab and the anti-GIgG activity of a G1 supernatant were titred by ELISA. The ELISA was carried out as described in section 2.8.1.1. Either a 10μg/ml solution of GIgG (to titrate the anti-GIgG activity) or a 10μg/ml solution of M6149 (to titrate the quantity of Mab) were used as coating antigens. Alkaline phosphatase conjugated to either goat anti-mouse IgM (Fig. 3.12), goat anti-mouse IgG (Fig. 3.13) or goat anti-mouse IgA (Fig. 3.14) were used as secondary antibodies, at the manufacturer's recommended dilution.*

This Mab showed equally good titres with the anti-μ and anti-γ secondary antibodies, ostensibly indicating that this Mab was a mixture of both isotypes, or possibly, not a true monoclonal antibody preparation. Another surprising aspect of this assay was the very low activity of this supernatant against GIgG.

was with IgG. Subsequently, positive results with both the anti-μ and anti-γ conjugates was taken as indicating that the Mab was of the μ isotype. All other Mabs were positive with the anti-μ conjugate (see Figs. 3.15-3.22) and negative with the anti-α conjugate (results not shown). Thus, all Mabs were considered to be of the μ isotype. Subsequently, all Mabs were isotyped with regard to their light chain using the same strategy as employed for their heavy chain (results not shown). These experiments clearly indicated that all Mabs were of the κ isotype.

With regard to Figs. 3.12 and 3.15-3.22 it was immediately apparent that the Mabs were broadly classifiable into two groups. G11 was the sole member of one group and showed considerable anti-GIgG activity. The remaining Mabs constituted the second group and showed low to non-existent (G3 and G30) anti-GIgG activities. The low-to-negative activities in the ELISA assay contrasted sharply with the positive activities obtained with the ELIspot assays used during the screening and cloning of these hybridomas. They were not due to low quantities of Mab in the supernatants (compare the ELISA titration curves, obtained for each supernatant, on M6149-coated wells). Thus, at first glance, these ELISA results appeared to contradict the ELIspot results and suggested that, none of the second group of Mabs were significantly reactive with GIgG.

This apparent contradiction was easily resolvable, however, if one was receptive to the possibility that binding sites may be polyreactive. To see why this is so, imagine 2 Mabs, equally reactive with GIgG, that is to say, the affinities of their respective interactions with GIgG are identical. However, one Mab is highly monoreactive; the second is highly polyreactive. Supernatants are highly complex matrices and in any hybridoma supernatant a dynamic equilibrium must exist between Mab, media components (MC) and Mab-MC complexes:

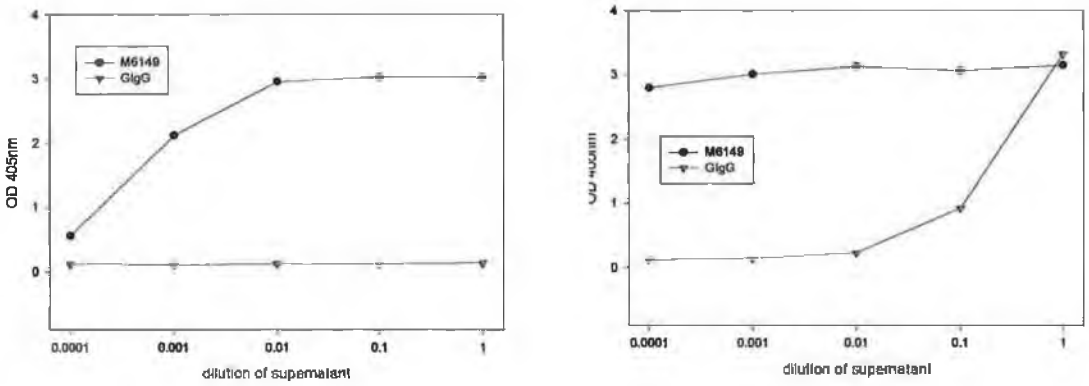
$$\text{Mab} + \text{MC} \rightleftharpoons \text{Mab-MC}$$

In the case of the monoreactive Mab, the equilibrium will exist far to the left. Hence, the anti-GIgG activity of the supernatant, which when detected by ELISA is dependent on the [Mab], will be high. In the case of the polyreactive Mab, the equilibrium may exist far to the right and, as a result, the anti-GIgG

activity of that supernatant may appear low-to-non-existent. Viewed from this perspective, the anti-GIgG activity of any supernatant must be considered a very poor indicator of the reactivity of the corresponding Mab with GIgG. Equally, this perspective suggests that the anti-GIgG activity as detected by ELIspot, is a much better indicator of the reactivity of the Mab with GIgG. This is due to the fact that, the ELIspot assay differs fundamentally from the ELISA in that, it detects freshly secreted antibody. This being the case, the dynamic equilibrium cannot be established to the right regardless of the nature of the binding site in question.

This interpretation of the results led to a number of working hypotheses. Firstly, the second group of Mabs were bound in solution by media components; these Mabs were tentatively classified as polyreactive. G11 was considered to be monoreactive. Secondly, if one could affinity-purify the polyreactive Mabs, they should show a higher specific anti-GIgG activity, as determined by ELISA, than their corresponding supernatants, as all the Mab in affinity-purified form should exist free in solution. Thirdly, if the polyreactive Mabs were bound in solution by media components, antigen affinity-purification would not be a useful means of purifying the Mabs. These hypotheses were tested and the results are reported in the section 3.3.

Fig. 3.21 **Fig. 3.22**



Figs. 3.15-3.22: *The quantities of Mab and the anti-GIgG activities of a series of supernatants from the G3, G4, G11, G18, G19, G24, G30 and G33 hybridomas were titrated by ELISA (figs. 3.15-3.22, respectively). The G11 supernatant was the only supernatant which showed significant anti-GIgG activity (see also fig. 3.12).*

ELISA: The ELISAs were carried out as described in the legend to Fig. 3.12-3.14, but only using an alkaline-phosphatase conjugated to goat anti-mouse IgM as a secondary antibody.

3.3 Analysis of Mab polyreactivity

At the start of this chapter, the hypothesis that IVI would result in polyreactive Mabs was advocated based on a review of the literature. This contention was strenghted by the fact, that a polyreactive rather than a monoreactive model of antibody binding sites, appeared better able to explain the data for 8 of the 9 hybridomas, presented in section 3.2.1. Reported in this section, are the results obtained using three different modes of analysis of the putative polyreactivity of the G1, G3, G4, G11, G18, G19, G24, G30 and G33 Mabs.

One approach used was to purify each Mab and compare its specific activity with that of its corresponding supernatant. An increase in specific activity was to be taken as indicative of a polyreactive binding profile. *(This approach was based on the notion that polyreactive Mabs should show a higher specific activity as their purity increases (see section 3.2.1). Although, it may be argued that the opposite argument (i.e. that an increase in specific activity is indicative of polyreactivity) does not necessarily hold, one can think of no other reasonable explanation for an increase in specific activity with increasing purity. This would be especially true if the percentage increase in specific activity was a characteristic peculiar to each Mab and, hence, most probably linked to its variable region).* Studies on IgM purification and specific activity determinations are reported in section 3.3.2. A second, more formal, approach used was to measure the affinity of the Mabs for a small panel of antigens, other than GIgG. Obviously, any detectable affinities were indicative of polyreactivity. This line of investigation is the subject of section 3.3.1. This approach was considered to be limited in its ability to discriminate between polyreactive and monoreactive antibodies, due to the limited numbers of antigens employed (see section 1.2.2). Thus, a third approach was used (reported in section 3.3.3), based on immunoblotting. In this approach, crude protein extracts are separated by SDS-PAGE and electrotransferred onto nitro-cellulose to provide an array of many hundreds of separated proteins. Antibodies, to be analysed for polyreactivity, are used as primary probes in an immunoblot of the nitro-cellulose. Thus, the antibodies are tested for their activity against many hundreds of antigens, simultaneously. Upon subsequent development of the immunoblot with a secondary antibody and substrate, the presence of bands indicate polyreactivity.

3.3.1 Method of Friguet *et al.* (1985): An ELISA-based approach originally published by Friguet *et al.* (1985) was used to measure the affinity of the Mabs for GIgG and a panel of 6 other antigens (DNP-human albumin (DNP), thyroglobulin (Thyr.), phosphorylcholine (PC), single-stranded DNA (ssDNA), bovine insulin (BI) and bovine serum albumin (BSA)). The theory of this method is fully described in the above reference. Briefly, an unknown but constant concentration of antibody is incubated with known, varying, concentrations of the antigen of interest. At equilibrium, a certain fraction of the antibody (ν) will be bound in solution by the antigen. This fraction is dependent upon the concentration of antigen ([A]). The following equation can be shown to hold:

$$\nu^{-1} = K_D [A]^{-1} + 1 \qquad \text{Eq. 3.1}$$

where K_D is the equilibrium constant for the interaction. Thus, a Klotz plot (ν^{-1} versus $[A]^{-1}$) should be a straight line giving a y-intercept of 1. The slope of the 'best-fit-line' is equal to the equilibrium constant for the interaction.

In order to calculate K_D, one must be able to calculate the values of ν^{-1} (as the values of [A] are at the discretion of the experimenter, they will be known). This is easily accomplished. Bear in mind that, if one can calculate the concentration of free antibody remaining in each equilibrium solution, one can calculate ν and, hence, ν^{-1}. The concentration of free antibody can be easily determined. As mentioned in section 3.2.1, the anti-GIgG activity of a solution (quantified by an ELISA as an OD 405nm measurement) is related to the concentration of free antibody in that solution. It should, thus, be self-evident, that the values of ν^{-1} can be obtained by determining the anti-GIgG activity remaining in each antibody-antigen mixture at equilibrium. This activity is then related to the concentration of free Mab by reference to a standard curve of optical density versus concentration of free antibody. The application of this technique to determine the equilibrium constant for the interaction between G11 Mab and GIgG is explained in more detail in section 3.3.1.1.

Two points must be borne in mind, if this technique is to be used correctly. Firstly, during the ELISA, the equilibrium established in solution will be interfered with, as free antibody in solution becomes bound to the solid phase. It is necessary that this interference be minimised, by ensuring that only a small fraction of free antibody in solution binds to the solid phase. It is possible to experimentally determine that this is so (Fig. 3.23). Secondly, it should be noted, that there is one assumption inherent in the mathematical treatment leading to equation 3.1. This is that the concentration of antigen is at least 10 times greater than that of the antibody. This was not formally proved. Nonetheless, this condition is likely to have been fulfilled. This is based on the assumption that, the antibody concentrations in the supernatants are 10μg/ml as conventionally found. For IgM, this roughly translates as a molarity of $1x10^{-8}$M. As all supernatants were used at a dilution of at least 1:10, in practice, a concentration of antigen greater than or equal to $1x10^{-8}$M was required. This condition was met for all experiments.

In total, this technique was used to characterise the interaction between 63 different antibody-antigen pairs (9 antibodies and 7 antigens). The data obtained is presented in section 3.3.1.1 and, in section 3.3.1.2, this data is used to classify the Mabs as polyreactive or otherwise.

3.3.1.1 **Affinity determinations:** Only for 17 of the 63 interactions analysed, were the interactions strong enough to characterise. For the particular interaction of G11 Mab with GIgG, Figs. 3.24-3.25 and Table 3.1 explain in great detail, the raw data obtained, and the subsequent data manipulation required, to derive the Klotz plot from which the K_D value for this interaction was determined. For the remaining 16 interactions, only the deduced Klotz plots are presented (Figs. 3.26-3.41). The derived affinity constants, characterising all 63 interactions, are listed in Table 3.2.

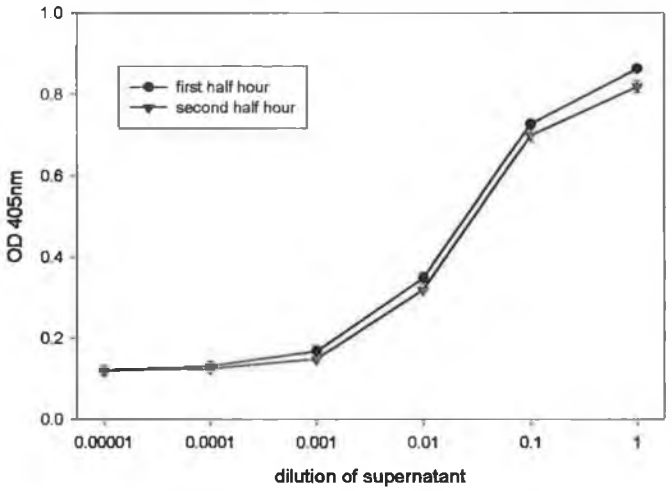


Fig. 3.23: *The anti-GIgG activity of a G11 supernatant was titrated by ELISA. Post-ELISA, the anti-GIgG activity remaining in the supernatant was determined. The accompanying graph depicts the activity (quantified as an OD 405nm measurements) of the supernatant before ('first half-hour') and after ('second half-hour') the ELISA assay. Both titration curves were very similar. It was inferred from this that, only a small fraction of the free Mab in solution had become bound to the solid phase during the ELISA.*

It should be noted that, it was only necessary to demonstrate for G11, that a small fraction of the solution phase antibody became bound to the solid phase during the ELISA. That this held true for the remaining Mabs, was amply proved by Figs. 3.12 and 3.15-3.22.

ELISA: The anti-GIgG activities were determined by ELISAs, essentially as described in section 2.8.1.1, but the test samples were only incubated in the wells for 0.5 hrs. GIgG was used at 10μg/ml as the coating antigen. An alkaline phosphatase conjugated to goat anti-mouse IgM was used as the secondary antibody at the manufacturer's recommended dilution.

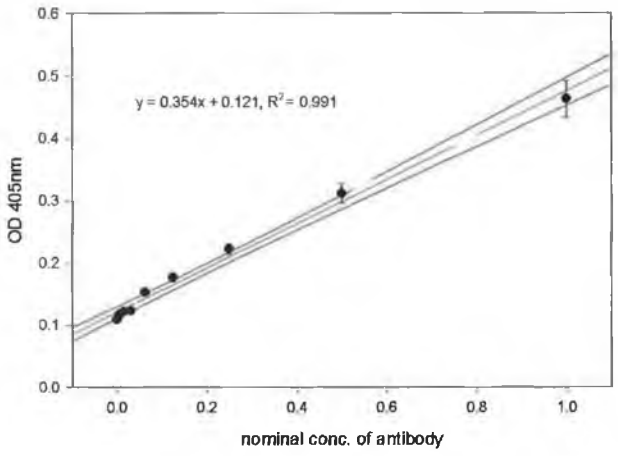


Fig. 3.24: *To utilise the method of Friguet et al. (1985) to determine the equilibrium constant for the interaction of G11 Mab with GIgG, one required a standard curve relating anti-GIgG activities to concentrations of free Mab.*

The concentration of free Mab in a G11 supernatant was unknown. To simplify this discussion, its concentration in a 1:100 dilution of the supernatant was referred to as a 'nominal concentration' of 1. A series of dilutions of the supernatant were prepared to give final nominal concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, 0.00390625 and 0. The anti-GIgG activity in each dilution was assayed in triplicate by ELISA and Fig. 3.24 depicts the resulting optical densities as a function of concentration of free antibody. (Each point represents the mean ± standard deviation of the triplicate measurements).

A linear relationship was expected and obtained between OD 405nm values and the free antibody concentration. The red line represents the best-fit-line; the black lines represent the corresponding 95% confidence intervals. The equation of the regression line is shown within the graph. This equation could then be used to determine the ***concentration of free G11 Mab in any solution, from an ELISA carried out in an identical fashion to that used to generate the standard curve.***

ELISA: The basic ELISA protocol of section 2.8.1.1 was used, but test samples were incubated for 0.5 hrs. rather than the stated 5 hrs. GIgG was used as the coating antigen at 10μg/ml and an alkaline phosphatase conjugated to a goat anti-mouse IgM was used as the secondary antibody at the manufacturer's recommended dilution.

$[GIgG]^{-1}$ (M^{-1}) $([A]^{-1})$	OD 405nm	NC*	$NC_0^{\dagger}/[NC_0-NC]$ (v^{-1})
$3.2x10^6$			
$6.4x10^6$			
$1.28x10^7$	0.161	0.113	1.14
$2.56x10^7$	0.182	0.172	1.23
$5.13x10^7$	0.3	0.505	2.24
$1.02x10^8$	0.399	0.717	4.71
$2.04x10^8$	0.405	0.802	8.42
$4.08x10^8$	0.421	0.847	14.44
$8.16x10^8$	0.423	0.853	15.96
∞	0.443	0.91	

* Nominal concentration of free antibody

† Nominal concentration of free antibody in the absence of antigen.

Table 3.1: *A series of G11 Mab-GIgG mixtures were prepared in triplicate, and allowed to equilibrate for at least 12 hrs. Each solution contained Mab at a nominal concentration of 1. Solutions contained different concentrations of GIgG as listed in the table.*

The following morning, the nominal concentration of free antibody, remaining in each mixture, was determined by an ELISA assay, carried out in an identical fashion (actually, at the same time and in the same plate) as that used to generate the standard curve of Fig. 3.24. The mean OD 405nm values obtained from the triplicate measurements are shown as a function of GIgG concentration in the above table. The equation of the regression line of the standard curve depicted in Fig. 3.24 allowed one to convert these mean values into nominal concentration values.

It was then possible to calculate v^{-1} from these nominal concentrations.

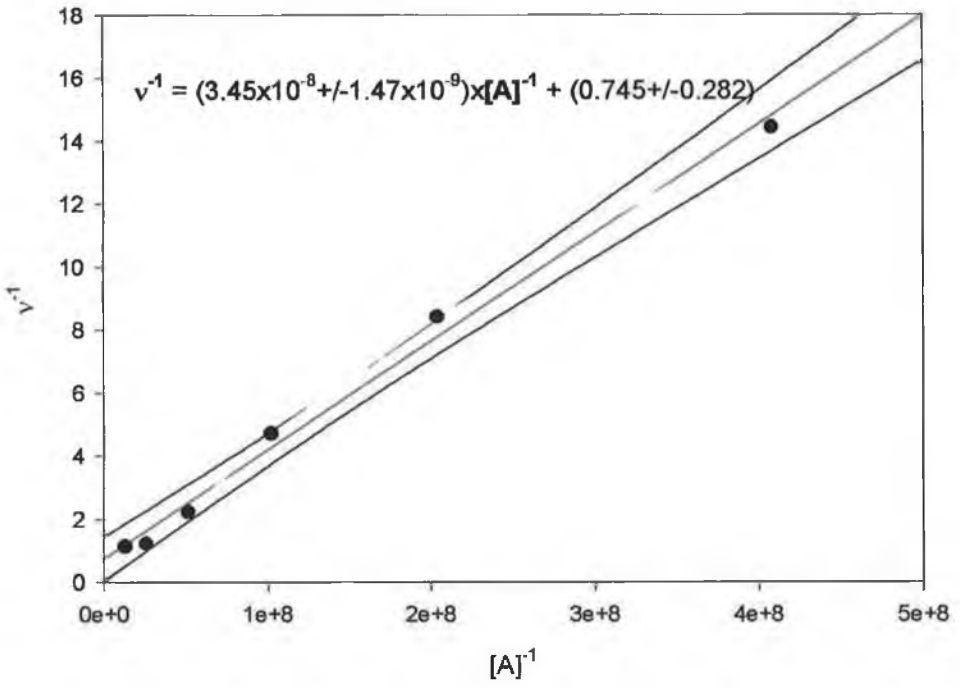


Fig. 3.25: *The Klotz plot (v^{-1} versus $[A]^{-1}$) used to characterise the interaction of GIgG with G11 Mab is shown above. The values of $[A]^{-1}$ and the corresponding values of v^{-1} were obtained from Table 3.1. The equation of the regression line gives the affinity of the interaction as* ***3.5 (± 0.1) x10^{-8} M.***

Fig. 3.26: G1 Mab-GIgG

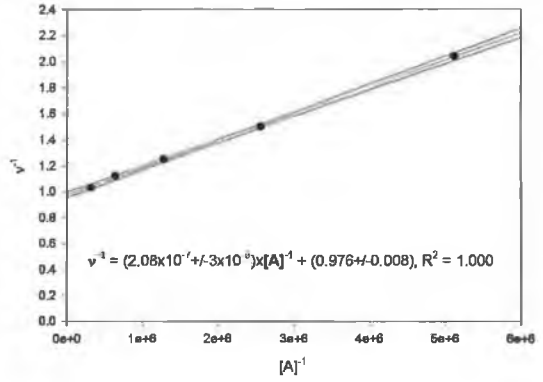


Fig. 3.27: G3 Mab-GIgG

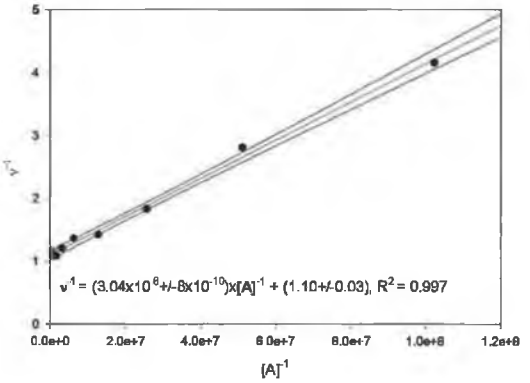


Fig. 3.28: G4 Mab-GIgG

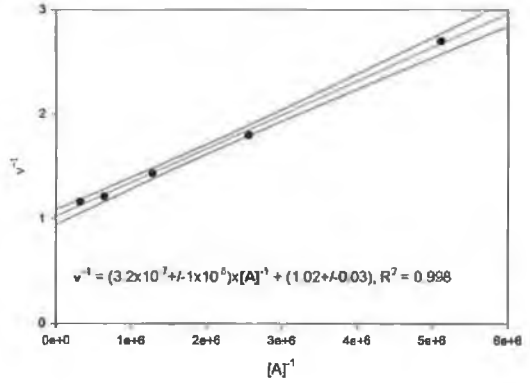


Fig. 3.29: G18 Mab-GIgG

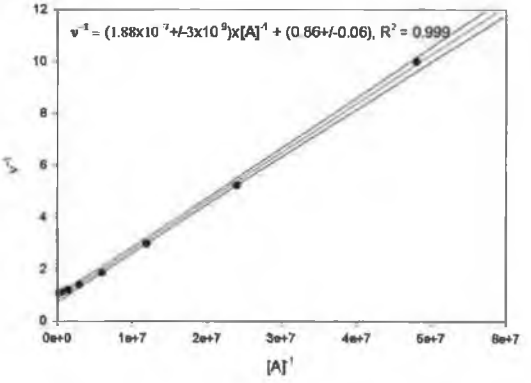


Fig. 3.30: G19 Mab-GIgG

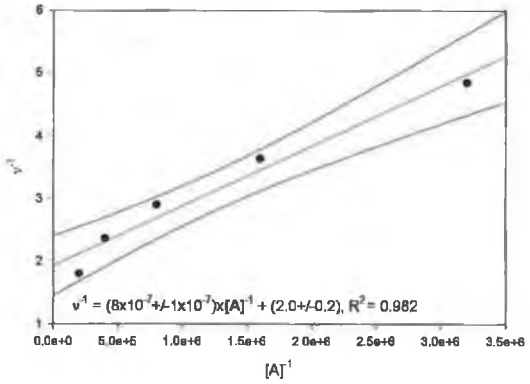


Fig. 3.31: G24 Mab-GIgG

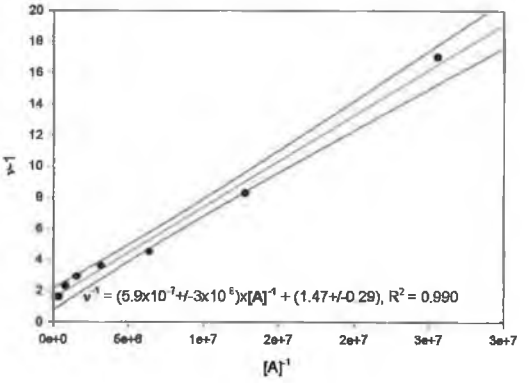


Fig. 3.38: G1 Mab-Thyr.

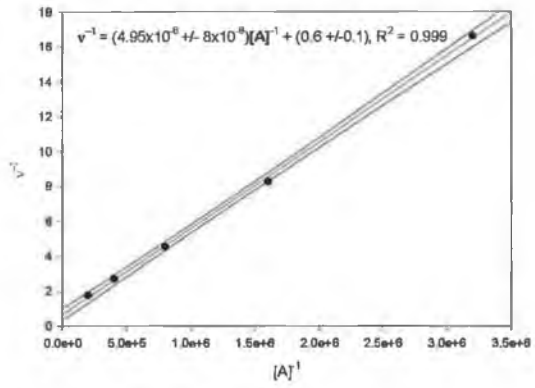


Fig. 3.39: G18 Mab-Thyr.

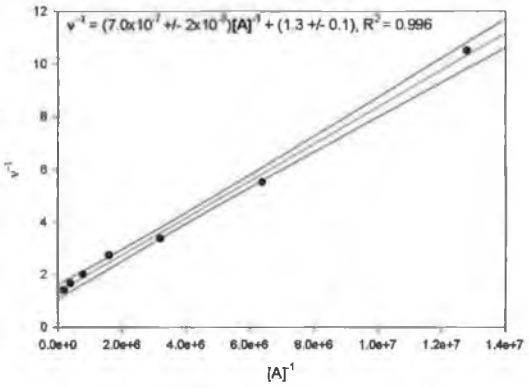


Fig. 3.40: G24 Mab-Thyr.

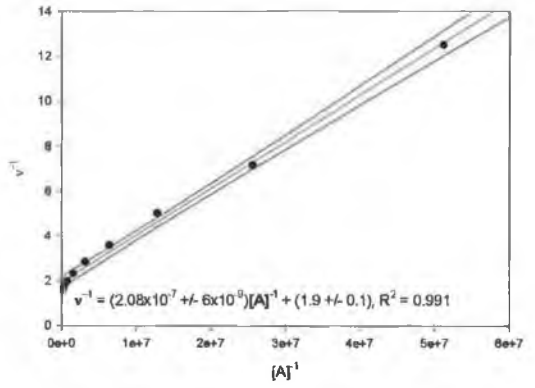
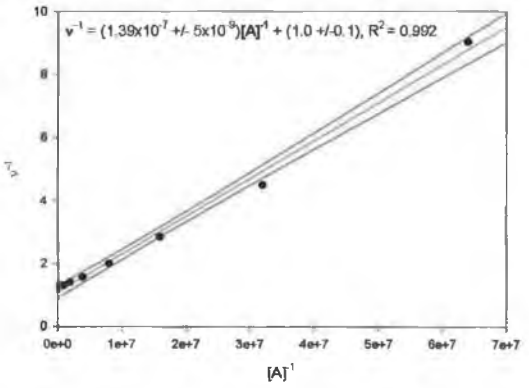


Fig. 3.41: G4 Mab-BI



Figs. 3.26-3.41: *The Klotz plots (v^{-1} versus $[A]^{-1}$) used to deduce the equilibrium constants for the interactions between a variety of antibody-antigen pairs are presented. The details of the antibody-antigen pairs are provided with each graph. GIgG: goat IgG, DNP: DNP-human albumin, Thyr.: thyroglobulin, BI: bovine insulin.*

	GIgG	DNP	Thyr
G1	2.08 (± 0.03) x10^{-7}	7.2 (± 0.8) x10^{-7}	4.95 (± 0.08) x10^{-6}
G3*	3.04 (± 0.08) x10^{-8}	<LOD	<LOD
G4	3.2 (± 0.1) x10^{-7}	<LOD	<LOD
G11	3.5 (± 0.1) x10^{-8}	<LOD	<LOD
G18	1.88 (± 0.03) x10^{-7}	3.65 (± 0.07) x10^{-8}	7.0 (± 0.2) x10^{-7}
G19	8 (± 1) x10^{-7}	2.9 (± 0.2) x10^{-8}	<LOD
G24	5.9 (± 0.3) x10^{-7}	9.6 (± 0.1) x10^{-9}	2.08 (± 0.06) x10^{-7}
G30*	3.1 (± 0.3) x10^{-8}	<LOD	<LOD
G33	7.7 (± 0.1) x10^{-8}	<LOD	<LOD

PC	ssDNA	BI	BSA
<LOD	<LOD	<LOD	<LOD
<LOD	<LOD	<LOD	<LOD
<LOD	<LOD	1.39 (± 0.05) x10^{-7}	<LOD
<LOD	<LOD	<LOD	<LOD
<LOD	<LOD	<LOD	<LOD
<LOD	<LOD	<LOD	<LOD
<LOD	<LOD	<LOD	<LOD
<LOD	<LOD	<LOD	<LOD
<LOD	<LOD	<LOD	<LOD

* The affinity data for these Mabs were calculated from affinity-purified Mabs (see section 3.3.2) rather than hybridoma supernatants.

Table 3.2: *The equilibrium constants characterising the interaction between 63 antibody (grey)-antigen (purple) pairs (9 x 7) are listed in the above table. These constants are listed as the mean ± standard deviation and are measured in terms of 'Molarity' (M). <LOD refers to calculated affinities, where the mean minus three times the standard deviation was less than zero.*

GIgG: goat IgG, DNP: DNP-human albumin, Thyr: thyroglobulin, PC: phosphoryl choline, ssDNA:single-stranded DNA, BI: bovine insulin, BSA: bovine serum albumin.

3.3.1.2 **Affinity measurements and polyreactivity:** The data presented in Table 3.2 clearly and formally indicated, that G1, G4, G18 and G24 were polyreactive. They reacted with at least one of the six antigens (GIgG was excluded from the analysis as this was the antigen against which the Mabs were raised) against which they were tested. However, the data is equivocal with regard to whether the remaining Mabs were polyreactive. Although these Mabs did not react with any of the six antigens, Fig. 3.42 indicates that this may purely be due to chance. In short, this approach to polyreactivity analysis was not sufficiently sensitive, to rule out the possibility that the remaining Mabs were polyreactive.

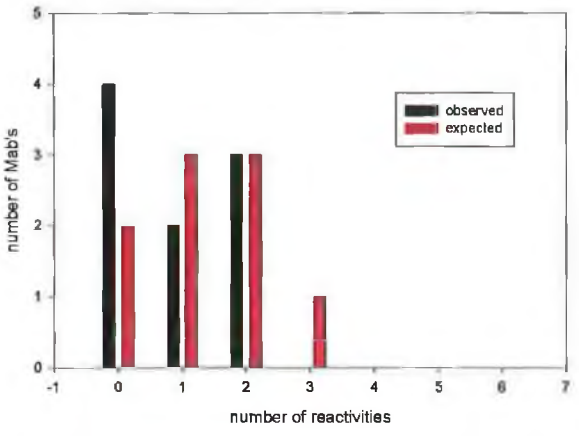


Fig. 3.42: *Based on the data presented in Table 3.2, Mabs were classified as reacting with from 0-6 of the antigens. They were reactive if their interaction with the antigen in question could be quantified by an equilibrium constant; otherwise they were unreactive. The above graph depicts the observed numbers of Mabs reacting with 0, 1, 2, 3, 4, 5, or 6 of the antigens.*

The question was then asked. Did this data support the null hypothesis, that the Mabs were equally polyreactive?

Under the null hypothesis, the expected numbers of Mabs reacting with from 0-6 antigens was calculated using a binomial model (Mabs were either reactive or not reactive) and 17/63 as the probability of an antibody reacting with an antigen. The 'goodness-of-fit' of the observed values with expected values cannot be calculated using a 'χ^2 goodness-of-fit test' as the frequency values were too low. Nonetheless, visually, there does not appear to be a huge difference between the observed and expected values. Thus, the null hypothesis could not be rejected.

3.3.2 The purification of Mabs and the determination of their specific activities:

The purification of monoclonal antibody from each of 9 hybridoma supernatants (G1, G3, G4, G11, G18, G19, G24, G30 and G33), was undertaken to test the postulate (section 3.2.1) that, with the exception of G11 Mab, the purified Mabs would have a higher specific anti-GIgG activity than their corresponding supernatants.

A number of approaches to IgM purification were conceivable. Firstly gel filtration, the conventional method used to purify IgM, was considered. It was rejected as it is most suited to situations of low volume/ high concentration. Supernatants from hybridomas do not meet this criterion. Also bovine IgM would co-purify. An attempt was made, however, to utilise ultrafiltration through a 300,000 Da cut-off membrane. The rationale for this was that in SDS-PAGE analyses of serum, the vast majority of serum proteins appear of 70,000 Da or less. However, attempts to purify IgM in this manner failed (results not shown) as the vast majority of serum proteins were retained by the membrane as indicated by SDS-PAGE analyses.

Affinity chromatography using any one of four different ligands was then considered. These ligands were mannose binding protein (MBP), protein L, GIgG and anti-mouse μ chain (anti-μ). A commercial MBP column was available for IgM purification. However, the purification procedure was not optimised for purification from tissue culture supernatants. Also some degree of uncertainty was felt with regards to the specificity of this ligand. This approach was rejected. Protein L binds specifically to κ light chains and was rejected for this very reason. It could not be used to purify an IgM antibody of the λ isotype.

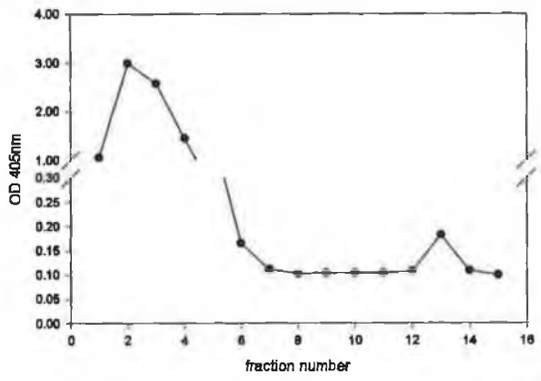
Although convinced that it would not work for reasons already discussed (section 3.2.1), an attempt was made to use a commercial GIgG-agarose column to purify the IgMs. 0.2ml of G1 supernatant was applied to the column, followed by 10ml of PBS and 5ml of elution buffer (0.1M glycine, pH 2.7). 15 x 1ml fractions were collected and fractions 11-15 (eluted fractions) were neutralised with 0.1ml of 1M Tris.Cl, pH 8.7, and dialysed overnight against PBS. The quantities of murine IgM and the anti-GIgG activities of each fraction were determined by ELISA (Figs. 3.43 and 3.44,

respectively). It was obvious that the major murine IgM peak and the only observable peak of anti-GIgG activity, which emerged from the column, centred about the second fraction. A SDS-PAGE analysis of each fraction (result not shown) indicated, that this was the fraction where the vast majority of the FCS-derived proteins were found. It was inferred from these observations, that the majority of the IgM passed straight through the column; only a small fraction was retained by the column and, subsequently, was eluted; hence the small quantity of mouse IgM in fraction 13.

While there is no theoretical reason why a monoreactive G1 Mab should pass through the column in such a bimodal fashion, this is exactly what one might expect if it was polyreactive. Using this model, it is to be expected that, at equilibrium, only a small fraction of the G1 Mab would remain free in the supernatant. This amount could bind to the affinity column and later appear in the eluate. The majority, however, would pass straight through the column and appear with the FCS-derived proteins. This experiment not only provided more supporting evidence for the view that the G1 Mab was polyreactive. It also indicated, given the belief that only G11 Mab was monoreactive, that an anti-μ column was the only sensible approach for the purification of these Mabs.

For this purpose, a commercial preparation of anti-μ was procured. For this to be an effective affinity ligand it was essential that, its interaction with IgM was regenerable. This was evaluated using the BIAcore™. This system was used for the simple reason that, it allows easy visualisation of interactions between biomolecules, within a short experimental timeframe.

Fig. 3.43 **Fig. 3.44**



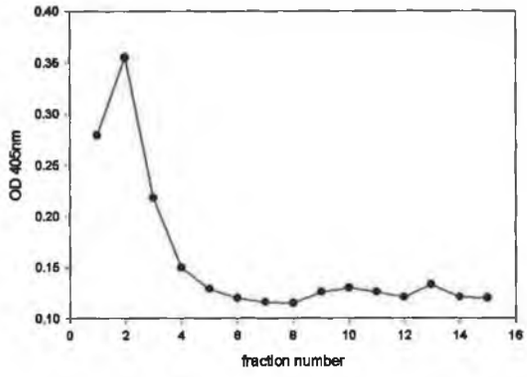


Fig. 3.43: *0.2ml of G1 supernatant was passed through a GIgG-agarose column. 10ml of PBS, followed by 5ml of elution buffer (0.1M glycine, pH 2.7) were passed through the column. 15 x 1ml fractions were collected and the quantities of murine IgM in each fraction were determined by ELISA, as described in section 2.8.1.1, with a 10μg/ml solution of M6149 as coating antigen. An alkaline phosphatase conjugated to goat anti-mouse IgM was used as the secondary antibody, at the manufacturer's recommended dilution. One major peak emerged from the column with PBS; a minor peak eluted off the column.*

Figs. 3.44: *The anti-GIgG activities in each of the 15 x 1ml fractions mentioned in the legend to Fig. 3.43, were determined by ELISA. The ELISA was carried out essentially as described in section 2.8.1.1, with a 10μg/ml solution of GIgG as coating antigen. An alkaline phosphatase conjugated to goat anti-mouse IgM was used as the secondary antibody, at the manufacturer's recommended dilution. All the observable anti-GIgG activity emerged from the column with the PBS.*

3.3.2.1 **BIAcore™ studies:** To allow an appreciation of the results obtained with the BIAcore™, the system will be briefly introduced. For a detailed introduction, however, the reader is referred to Jonnson *et al.*(1991).

The BIAcore™ is a commercially available biosensor, allowing 'real-time Biomolecular Interaction Analysis (BIA)'. The system makes use of specialised, disposable 'chips'. These chips, referred to as 'CM5 chips', contain a layer of carboxy-methylated dextran (referred to as the 'dextran matrix') attached to a gold layer. When inserted into the hardware of the system, they are juxtaposed with a flow cell, which allows one to pass solutions over and through the dextran matrix. Surface plasmon resonance, which is the basis for measurements with Biacore™, is an optical phenomenon arising in thin metal films under conditions of total internal reflection. This phenomenon produces a sharp dip in the intensity of reflected light at a specific angle (known as the resonance angle). This resonance angle is a function of the refractive index of the medium close to the non-illuminated side of the metal film. The Biacore™ measures the resonance angle (in terms of response units) as a function of time to produce a sensorgram. Changes in response units are then interpreted in terms of changes in the refractive index in the dextran matrix. Interpretation of sensorgrams will be introduced using Fig. 3.45 as a specific example.

For BIA, one of the pair of interacting molecules must be immobilised in the dextran matrix. In the experiment, which led to the sensorgram depicted in Fig. 3.45, anti-μ was immobilised. A fresh CM5 chip was inserted into the BIAcore™. An isotonic salt solution, referred to as 'running buffer', was passed over the surface. Running buffer is the default flow solution, and at position 1 in the sensorgram, running buffer is still flowing over the surface of the chip. A constant response (RU) is obtained because its refractive index is constant. This response is given a nominal value of 0 RU as all changes in response will be measured relative to this ***baseline***. At position 2, running buffer is again being passed over the surface. However, a ***net, permanent change in response*** of 147 RU is apparent. A ***sharp, transient change in response*** is evident, also, between position 1 and 2. This transient change is caused by a switch from running buffer to a solution of NHS/EDC. As this

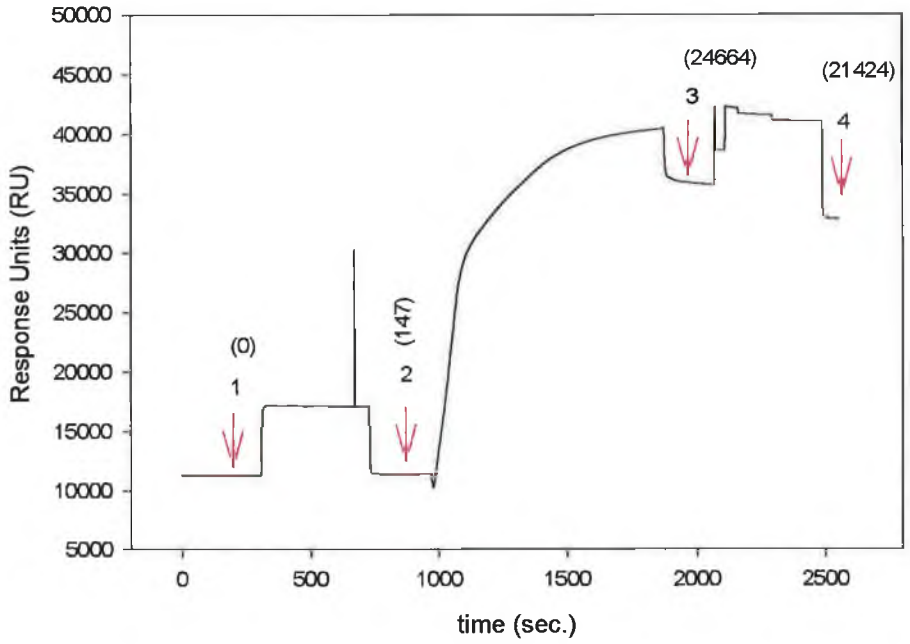


Fig. 3.45: *This sensorgram depicts the immobilisation of anti-μ to the dextran matrix of a CM5 chip. Between position 1 and 2, a NHS/EDC solution was passed over the chip surface, to activate the carboxy-methyl groups of the dextran matrix. This resulted in a small change in response, relative to position 1 (the baseline), of 147 RU. Between positions 2 and 3, a 50μg/ml solution of anti-μ was passed over the surface. Anti-μ became bound to the carboxy-methyl groups of the dextran matrix, via its amine groups; hence, a large change in response of 24,664 RU was observed. Finally, between positions 3 and 4, a solution of 0.1M ethanolamine, pH 9.0, was passed over the surface. This was to deactivate any remaining activated carboxy-methyl groups. A slight decrease in response was evident post-ethanolamine. This was attributed to removal of loosely adherent anti-μ, from the matrix, by the basic solution.*

solution has a different refractive index to running buffer, one gets a transient increase in response, which disappears upon reversion to running buffer. To understand the residual permanent change in response, which is unaffected by reversion to running buffer, one must understand the role of the NHS/EDC solution. This solution results in the chemical modification and activation of

the carboxy-methyl groups in the dextran. This modification permanently changes the refractive index in the matrix.

At position 3 running buffer is again passing over the dextran surface and a further ***net, permanent change in response*** of 25000 RU has accrued. Between position 2 and 3, a 50μg/ml solution of anti-μ in 0.01M NaAc, pH 4.7, was passed over the surface. Under these conditions, anti-μ is attached via its amine groups, to the activated carboxy-methyl groups, hence, immobilising it within the dextran matrix. Again, it is the resulting change in the chemical constitution of the dextran matrix that underlies the permanent change in response. All that is now required, prior to engaging in BIA with anti-μ, is deactivation of any remaining activated carboxy-methyl groups. This is achieved by passing a solution of 0.1M ethanolamine, pH 9.0, over the surface. In the sensorgram depicted in Fig. 3.45, this occurs between positions 3 and 4. The small decrease in response, evident post-ethanolamine, is due to this solution removing loosely bound anti-μ from the dextran matrix. This surface is now ready for BIA of the interaction of anti-μ, with its ligand, mouse IgM.

G1 supernatant was passed over this derivatised surface (Fig. 3.46). An increase in response of over 1000 RU was obtained, attributed to binding of IgM by the anti-μ. In an attempt to regenerate the surface (i.e. disrupt the binding of IgM by anti-μ), a series of acid glycine buffers were, sequentially, passed over the surface (Fig. 3.46). In order to evaluate the regenerative capacity of the various buffers one assumption was made. This was, that the change in response was due, solely, to changes in the mass of IgM bound in the dextran matrix. This assumption allowed one, to calculate the amount of IgM remaining in the dextran matrix using the following theoretically derived relationship. ***Any change in response due to changes in the mass of protein bound within the dextran matrix, is directly proportional to the mass change.*** The application of this model indicated, that even the harshest buffer appeared to leave 74% of the IgM bound by anti-μ. Thus, an initial analysis of these results appeared to confirm that this batch of anti-μ was not suited to the requirements of affinity purification. Subsequently, however, it was calculated that the 20000 RU of anti-μ immobilised corresponded to a concentration of

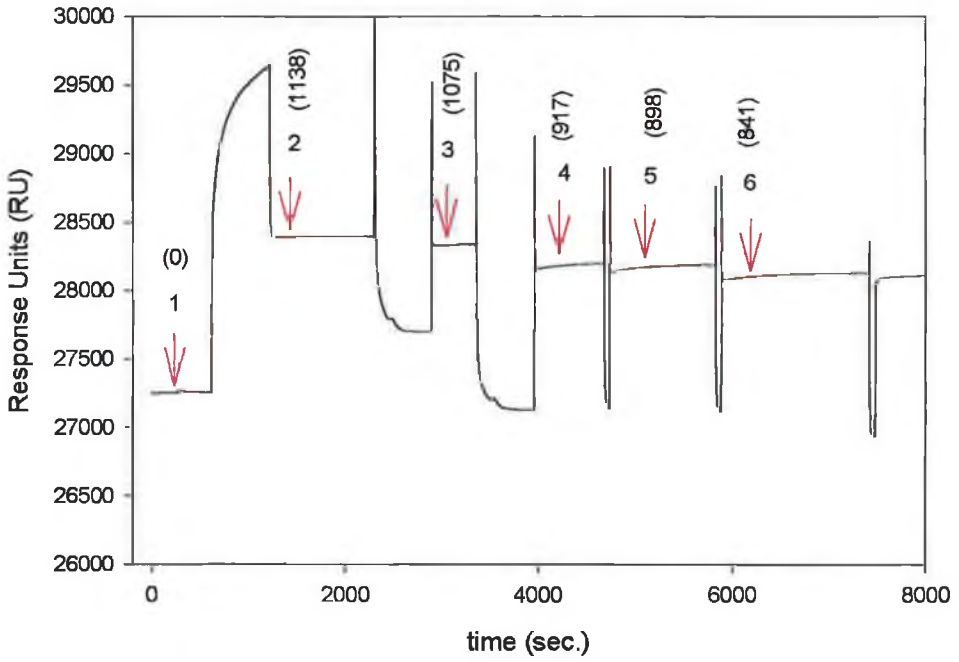


Fig. 3.46: *In the above sensorgram, G1 supernatant was passed over the anti-μ surface. This occurred between positions 1 and 2, and an increase in response of 1138 RU was obtained. This was attributed to binding of G1 Mab, by the anti-μ in the dextran matrix, as fresh G1 culture medium did not lead to any increase in response when passed over this surface. Between positions 2 and 3, 0.1M glycine, pH 2.9, was passed over the surface in an attempt to disrupt the interaction between the Mab and anti-μ. The response, relative to the baseline, decreases to 1075.* ***This decrease in response was attributed to a decrease in mass of Mab bound in the dextran matrix by anti-μ.*** *This simple model suggested that 94% of the Mab was still retained by the anti-μ. 0.1M glycine buffers (pH's 2.6, 2.4 and 2.2) were passed over the surface between positions 2 and 3, 3 and 4, and 4 and 5, respectively. Even using the harshest buffer, 74% of the Mab appeared to be retained by the anti-μ.*

anti-μ in the dextran matrix of > 100mg/ml. This did not accurately reflect the situation on affinity columns where in general proteinaceous ligand concentrations of ≤ 10mg/ml are used. It was considered that the high concentration of anti-μ in the dextran matrix may have contributed to the difficulty in regenerating the surface. Therefore, it was decided to make one more attempt at regenerating the interaction on the BIAcore™, by immobilising less anti-μ on the surface.

1000 RU of anti-μ were immobilised on a new chip surface (result not shown). As depicted in Fig. 3.47 and described in the corresponding legend, the interaction of IgM with anti-μ proved even more refractory to regeneration in this experiment, than the initial experiment, contrary to expectation. Not only did the sensorgram suggest that no regeneration was taking place, it also indicated an increase in RU after passing 0.1M glycine, pH 2.2, over the surface. This apparent increase in refractive index was inexplicable, in terms of the assumption that changes in response were due, solely, to changes in mass of bound protein in the dextran matrix. Some other factor was obviously causing an increase in response and, provisionally, this was attributed to a contraction of the dextran matrix upon exposure to this particular regeneration buffer. Regardless of the exact cause, it was obvious that the model being used to evaluate the extent of regeneration was flawed. Most likely, it was underestimating the extent of regeneration. In order that the model could be successfully applied, it was decided to use a different low pH solution, in the hope that, it would indicate a greater regenerative effect of low pH on the protein interaction.

As indicated in Fig. 3.48, approximately 2000 RU of anti-μ was immobilised on a fresh CM5 chip, G1 supernatant was passed over the chip, and an increase in response of 215 RU was obtained. 10mM, 20mM, 30mM and 40mM HCl (corresponding to pH's of 2.25, 2.15, 2 and 1.85, respectively) were successively passed over the surface (Fig. 3.48). 10mM HCl did not result in any regeneration. 20mM HCl apparently only left 18% of the IgM on the surface. After 30mM HCl only 8% of the IgM appeared to remain upon the surface and this was reduced to 5% after passing 40mM HCl over it. This

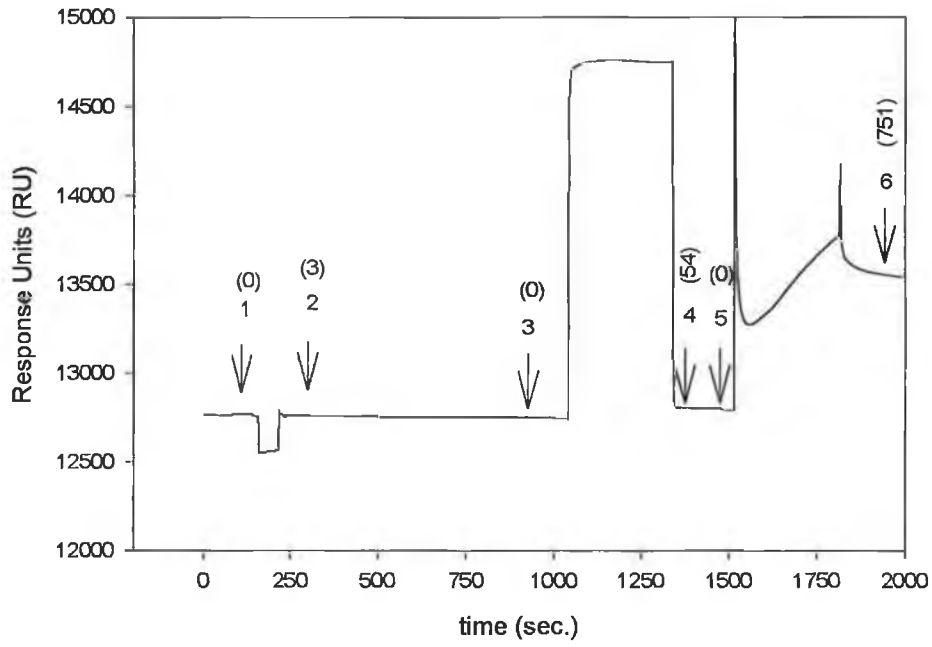


Fig. 3.47: *1000 RU of anti-μ were immobilised on a fresh CM5 chip, and 90 RU of Mab was bound by it, from G1 supernatant, prior to running this sensorgram. Between positions 1 and 2, 0.1M glycine, pH 2.2, was passed over the surface. Rather than the expected decrease in response, due to disruption of the anti-μ : Mab interaction, a slight increase in response was noted. Between positions 2 and 3, G1 supernatant was, again, passed over the surface. A slight increase in response, of 54 RU, was noted, due presumably to binding of Mab by the anti-μ. Between positions 3 and 4, 0.1M glycine, pH 2.2, was passed over the surface. Unexpectedly, this led to a very large increase in response of 750 RU, which was inexplicable in terms of changes in the mass of protein bound within the dextran matrix.*

experiment led one to conclude that, low pH solutions could be used to regenerate the particular interaction being analysed.

However for the anti-μ to be an effective affinity ligand, it was necessary to demonstrate that it retained activity after exposure to low pH. To evaluate retention of activity, 1500 RU of anti-μ was immobilised on the chip surface. Then the analyte (G1 supernatant) followed by the regeneration solution (40mM HCl) were successively and repeatedly passed over ('cycled') the surface. The responses post-analyte and post- regeneration were plotted (Fig. 3.49) as a function of cycle number. Although the post-regeneration response did increase with cycle number, anti-μ activity (i.e. post-analyte response) appeared to be completely retained even after 7 regeneration cycles. The increase in the baseline response was probably attributable to retention of some IgM by anti-μ after each cycle.

Finally, the effect of low pH on the immunoreactivity of IgM itself was analysed. To do this, 5000 RU of normal mouse IgM was immobilised on a chip surface. Analyte (10μg/ml solution of anti-μ in PBS) and 30mM HCl were successively cycled over this surface. The responses post-analyte and post-regeneration are depicted in Fig. 3.50. A one-off decrease in the immunoreactivity of the surface was evident after the first cycle but, thereafter, the immunoreactivity remained constant.

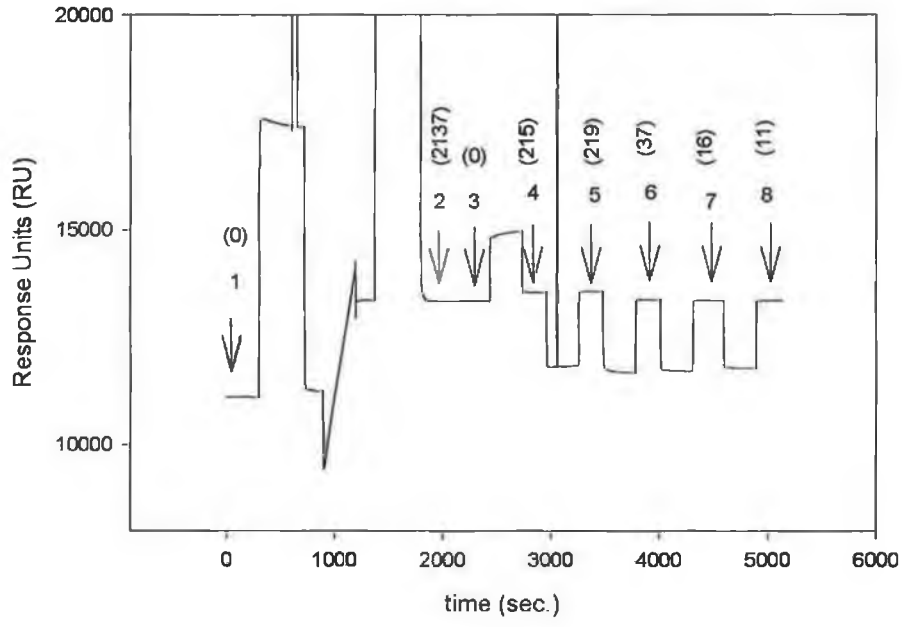


Fig. 3.48: *This sensorgram depicts the immobilisation of 2000 RU of anti-μ, on a CM5 chip surface. G1 supernatant was then passed over the surface and 215 RU of Mab was bound by the anti-μ. 10mM, 20mM, 30mM and 40mM HCl were then successively passed over the surface. 40mM HCl appeared to leave only 5% (11/215) of the Mab bound by the anti-μ. This was taken as indicating that, this interaction could be successfully regenerated using low pH solutions.*

Fig. 3.49 **Fig. 3.50**

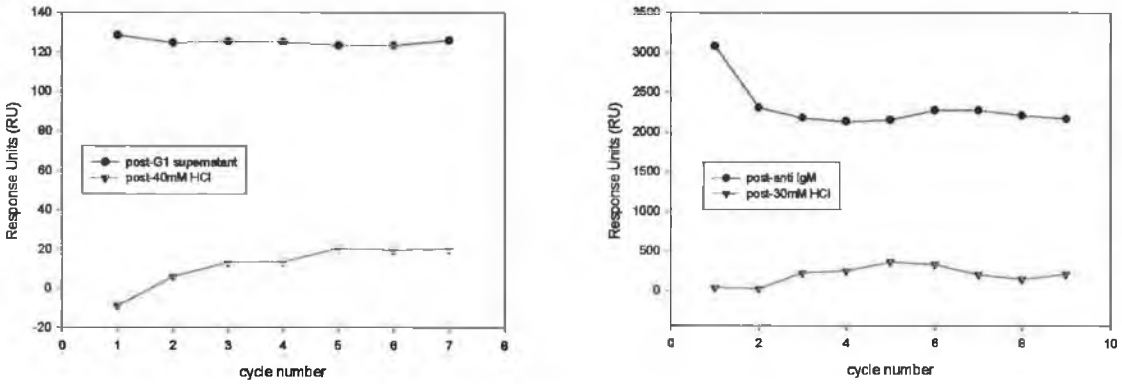


Fig. 3.49: *Retention of anti-μ activity, after exposure to low pH, was evaluated by successively cycling analyte (G1 supernatant) and regeneration solution (40mM HCl) over a dextran matrix containing immobilised anti-μ. The responses were measured post-analyte and post-regeneration and plotted as a function of cycle number. There was no apparent reduction in anti-μ activity as indicated by the constant post-analyte response.*

Fig. 3.50: *The retention of mouse IgM activity after exposure to low pH was evaluated by successively cycling analyte (anti-μ) and regeneration solution (30mM HCl) over a dextran matrix containing immobilised mouse IgM. A one-off decrease in the activity of the IgM was evident, in that, there was a decrease in the analyte response between the first and second cycle. Thereafter the post-analyte response was more-or-less constant.*

3.3.2.2 **Creation of an anti-μ affinity column:** The studies described in section 3.3.2.1 were sufficiently successful to make affinity purification of IgM, using anti-μ as the capture ligand, an attractive proposition. The basic protocol of section 2.8.5 was used to immobilise anti-μ on a Sepharose 4B solid phase. 3g of Sepharose 4B was pre-swollen and added to 6mg (determined spectrophotometrically) of anti-μ dissolved in 5ml of coupling buffer. After incubation of the suspension for 36 hrs, a spectrophotometric analysis indicated that only 58% of the anti-μ had bound to the Sepharose. The reason for this low value was not readily apparent. The 10ml of wet gel, subsequently, was packed in a commercial chromatography column.

3.3.2.3 **IgM purification:** Prior to purification, supernatants from all hybridomas were harvested and concentrated as per section 2.8.4.1. This concentrate was referred to as supernatant (conc.). In the cases of G1, G4, G11 and G18, hybridomas were adapted to grow in a serum-free formulation and supernatants were harvested, only when the percentage of FCS in the media was less than 0.01% (v/v).

Initially, an attempt was made to purify IgM from 10ml of G11 supernatant (conc.). This was passed through the affinity column followed by 20ml of PBS. Elution of retained protein was attempted with 20ml of 0.1M glycine buffer, pH 2.2 followed by 10ml of 0.1M glycine, pH 1.85; eluate was collected in 1 ml fractions. A total of 35 fractions were collected and 0.1 ml of neutralising buffer (1M Tris.Cl, pH 8.7) was added to each. The quantity of protein in each fraction was then determined spectrophotometrically at 280nm (Fig. 3.51). A rather broad protein peak, which tailed-off rather slowly, eluted of the column. This was probably due to the geometry (short, fat) of the column used. Notably, only one peak appeared to elute off the column. This was taken to indicate that, the vast majority of the IgM eluted with the 0.1M glycine, pH 2.2, and it was determined not to use the pH 1.85 solution in future purifications.

Fig. 3.51

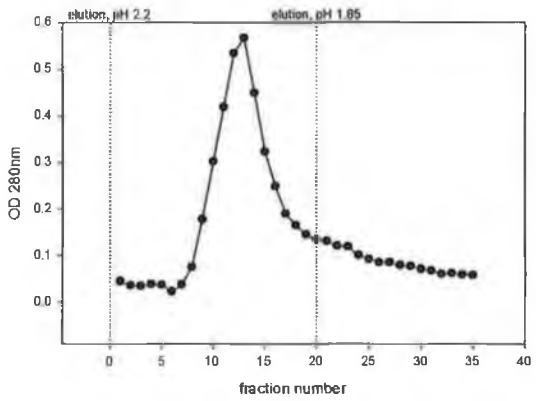


Fig. 3.52

Fig. 3.51: *G11 supernatant was passed through the anti-μ-Sepharose 4B column, and retained protein was eluted with 20ml of 0.1M glycine, pH 2.2, and 15ml of 0.1M glycine, pH 1.85. The eluate was collected in 35 x 1ml volumes and the quantity of protein in each fraction was determined spectrophotometrically at 280nm. One protein peak eluted off the column, with a substantial degree of tailing. This was attributed to the column geometry (short and broad column).*

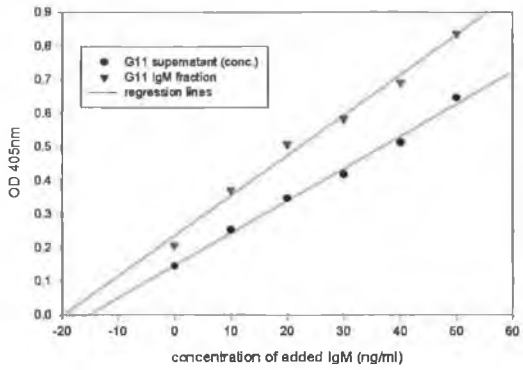
Fig. 3.52: *The IgM fractions purified from G19, G18, G11, G4, G3 and G1 (lanes 1-6 respectively) were run through a SDS-PAGE gel, under reducing conditions, and stained with Coomassie blue. Molecular weight (Mw) markers were also run: Carbonic anhydrase (29,000 Da), Ovalbumin (45,000 Da), Bovine serum albumin (66,000 Da), Phosphorylase b (97,400 Da), β-galactosidase (116,000 Da) and Myosin (205,000 Da). The banding-patterns obtained for the IgM fractions from G1 and G3 appeared very faint and could not be interpreted. Nonetheless, the remaining IgM fractions show two clear bands, which occurred where one would expect to find polypeptides of ≈ 23,000 Da and ≈ 70,000 Da. These were tentatively identified as an antibody light chain, and a μ heavy chain, respectively. There was no indication of a J chain.*

Fractions 11-17 were pooled and dialysed overnight against PBS containing 0.5M NaCl. This fraction was referred to as the IgM fraction. This fraction (along with a number of IgM fractions purified from the supernatants of other hybridomas) were analysed for purity by SDS-PAGE as per section 2.8.3.2 (Fig. 3.52). For most of the IgM fractions, two bands were apparent upon staining with Coomassie blue. They roughly correspond to the molecular weight of a μ-chain (≈70,000) and a light chain (≈23,000). No band was evident where one would expect to find a J-chain. This was presumably due to its low abundance and, possibly, its staining characteristics.

In order to evaluate and compare the specific anti-GIgG activity in the G11 supernatant (conc.) and the IgM fraction, the concentration of Mab in both was determined, by standard addition. Standard addition refers to an analytical technique, where a series of known amounts of analyte are added to the unknown. In each case, the total amount of analyte (unknown plus standard) is quantitated as, e.g., an absorbance value. A graph of absorbance versus concentration of added standard is plotted. The absolute value of the x-intercept of the regression line is equal to the concentration of unknown (Harris, 1991). The main advantage of this technique is that the matrix remains constant for all samples. Using this technique, the concentrations of Mab in both the G11 supernatant (conc.) and its corresponding IgM fraction were determined as 1.5mg/ml and 0.39mg/ml, respectively (Fig. 3.53).

The anti-GIgG activities of both samples were titrated by ELISA, and plotted as a function of concentration of Mab (Fig. 3.54). Specific anti-GIgG activity, then, was defined as the slope of the regression line to the linear portion of a plot of anti-GIgG activity, versus concentration of Mab. The specific anti-GIgG activity of the supernatant (conc.) was 0.0337 ± 0.0007 ml/μg and that of the IgM fraction was 0.034 ± 0.002 ml/μg. These specific activities were almost identical. This led one to the conclusion that the affinity purification had been very successful, as it was accomplished without any apparent diminution in Mab activity.

Fig. 3.53 **Fig. 3.54**



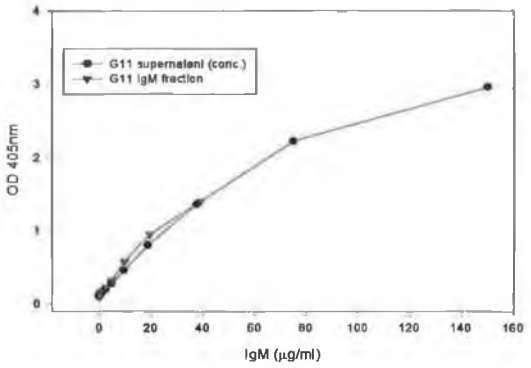


Fig. 3.53: *A 1:100000 dilution of G11 supernatant (conc.) and a 1:20000 dilution of its corresponding IgM fraction were prepared. The quantities of Mab in both unknowns were determined by standard addition, to the unknowns, of a series of known amounts of normal mouse IgM (0-50ng/ml). The total amount of mouse IgM, in each sample, was quantified by ELISA, and plotted versus the concentration of added standard.*

The absolute value at the intersection of the regression line with the x-axis, was taken as the concentration of IgM in the unknown. For the 1:100000 dilution of G11 supernatant (conc.), this value was 15 ng/ml. Thus, the conc. of IgM in the undiluted G11 supernatant (conc.) was 1.5 mg/ml. The concentration of IgM in the corresponding IgM fraction was calculated as 0.39 mg/ml.

ELISA: This was carried out as described in section 2.8.1.1. A 10µg/ml solution of M6149 was used as the coating antigen, and an alkaline phosphatase conjugated to goat anti-mouse IgM was used as the secondary antibody, at the manufacturer's recommended dilution.

Fig. 3.54: *The anti-GIgG activities of both G11 supernatant (conc.) and G11 IgM fraction were titred by ELISA, and plotted as a function of Mab concentration. The specific activities of both the supernatant (conc.) (0.0337 ± 0.0007 ml μg^{-1}) and the IgM fraction (0.034 ± 0.002 ml μg^{-1}) were defined as the slopes of the regression lines to the linear portion of both curves.*

ELISA: as described in the legend to Fig. 3.53, but a 10µg/ml of GIgG was used as the coating antigen.

3.3.2.4 **Specific anti-GIgG activity as a measure of polyreactivity:** All the remaining Mabs were purified, as described in the previous section for G11 Mab. In each case, the quantities of Mab in both the supernatant (conc.) and corresponding IgM fraction were determined by standard addition, and the anti-GIgG activities, in both, titred by ELISA. The anti-GIgG activities were plotted versus concentration of Mab (see Fig. 3.55-3.62), and the specific anti-GIgG activities of each supernatant (conc.) and its corresponding IgM fraction were calculated, as described for G11 Mab (section 3.3.2.3). The specific activities are tabulated in Table 3.3.

It was immediately evident that, as predicted in section 3.2.1, all Mabs segregated in one of two groups. G11 Mab was the sole occupant of one group, distinguished by the similarity of its specific activity in supernatant (conc.) form to that as an IgM fraction. G11 Mab was said to be 'monoreactive' as a term-of-convenience. The remaining Mabs constituted the second group and were distinguished by the large increases in specific activity attendant upon purification. These Mabs were considered to be polyreactive.

Fig. 3.61 **Fig. 3.62**

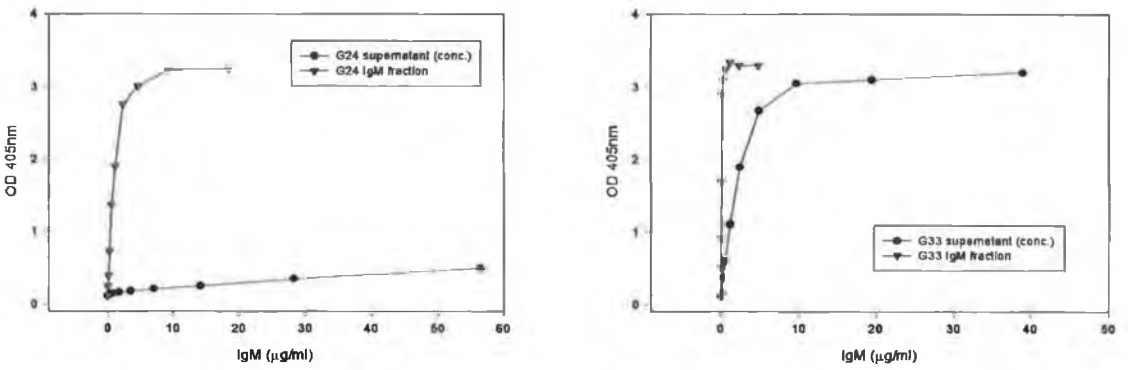


Fig. 3.55-3.62: *Mabs were purified from supernatants of the G1, G3, G4, G18, G19, G24, G30 and G33 hybridomas, as described in section 3.3.2.3. The quantities of Mab in each supernatant and its corresponding IgM fraction were determined by standard addition, as described in section 3.3.2.3, and their anti-GIgG activities were determined by ELISA. In each case, the anti-GIgG activity was plotted as a function of Mab concentration, and the specific activity of each solution was deduced from the slope of the regression line to the linear portion of each curve. These specific activities are tabulated in Table 3.3.*

	Specific activity of supernatant (conc.)	Specific activity of the IgM fraction	Percentage increase in specific activity
G1	0.0228 ± 0.0009	1.28 ± 0.09	5,500%
G3	<LOD	0.18 ± 0.02	ND
G4	0.0108 ± 0.0003	0.081 ± 0.003	650%
G11	0.0337 ± 0.0007	0.034 ± 0.002	0.9%
G18	0.066 ± 0.001	0.760 ± 0.007	1,050%
G19	0.0045 ± 0.0004	0.7 ± 0.1	14,500%
G24	0.0065 ± 0.0003	2.20 ± 0.03	33,700%
G30	0.015 ± 0.001	24.8 ± 0.5	164,300%
G33	0.84 ± 0.04	21.8 ± 0.23	2,400%

Table 3.3: *The specific activities of all hybridoma supernatants and their corresponding IgM fractions are depicted above (all measured in ml μg^{-1}). The increase in specific activity refers to that of the IgM fraction over the supernatant. This was calculated by dividing the mean specific activity of the IgM fraction by that of the corresponding supernatant.*

3.3.3 Immunoblots:

For the final test of polyreactivity-the immunoblot-it seemed sensible to use the affinity-purified Mabs rather than the hybridoma supernatants. In this form, all the binding-sites were considered to be free and could interact with the proteins immobilised on the nitro-cellulose. On the otherhand, in supernatant form, the percentage of Mab, which remained free, was probably very limited. For example, the specific activity determinations for G30 Mab (Table 3.3) suggested that, only, 0.06% (0.015x100/24.8) of the total Mab remained free in the supernatant. This sequestration was expected to be very deleterious to the sensitivity of the assay.

In an initial experiment, G1, G4, G11, G18 and G19 affinity-purified Mabs were used to probe a template of size-fractionated liver proteins, immobilised on nitro-cellulose. Two purified mouse IgM monoclonal antibodies, produced independently of the studies reported in this chapter, were also used to probe this blot, in the expectation that they would serve as negative controls. Clone E51.87 is an anti-CD5, produced by *in vivo* immunisation (Mark *et al.,* 1981). Clone 11E10 is an anti-lipopolysaccharide IgM, also produced by *in vivo* immunisation (Southern Biotechnology Technical Division, personal communication). Upon development with secondary antibody and substrate, the blot depicted in Fig. 3.63 was obtained. The result was very unexpected. All the Mabs produced for this study, *including G11*, appeared to be polyreactive in that, they all resulted in a similar multi-band staining pattern. Clones E51.87 and 11E10, while not completely negative on this blot, at least gave much less intense staining patterns, than the Mabs generated by IVI. This evidence in favour of a polyreactive G11 Mab was directly opposed to the evidence, presented in section 3.3.2, of the specific activity determinations.

In an attempt to resolve this apparent contradiction, it was noted that, the intensity of the staining pattern developed on the blot was related to the harshness of the procedure used to purify the Mab. All the Mabs generated during the course of this study had been purified by elution from an affinity matrix, using low pH. The remaining two Mabs had been purified, however, by size-exclusion chromatography followed by anion-exchange chromatography -

a much gentler procedure. It was considered possible that, the exposure to low pH had in some way disturbed in a minor fashion, the binding sites of each Mab. In the case of G11 Mab, this had converted a monoreactive Mab into, ostensibly, a polyreactive one. To test this, advantage was taken of the similar specific activities of G11 supernatant and its corresponding IgM fraction, both of which were used to probe a template of liver proteins resulting in the blot of Fig. 3.64. This experiment clearly revealed, that exposure to the low pH had changed the binding-characteristics of this Mab, at least as revealed by the immunoblot assay. Quite clearly, one had no choice if one wanted to avoid artifactual observations, but to use the hybridoma supernatants for this assay.

These concentrated supernatants were used to probe a template of size-fractionated liver proteins and the blot, which resulted, is presented in Fig. 3.65. There are a number of noteworthy aspects to this blot. Firstly, the staining patterns observed were the result of binding by the Mabs to liver proteins. Two observations lead to this conclusion. The first is that the secondary antibody only weakly reacted with the liver proteins (only a weak staining pattern was evident in regions of the blot between the lanes in which the supernatants were run). The second is that although the staining patterns observed in each lane are similar, in terms of the bands stained, the relative intensity with which they are stained is a characteristic peculiar to each supernatant.

The second noteworthy point is that the G11 Mab was the only Mab, which did not react with the template of liver proteins. It is true that many of the other Mabs reacted weakly on the blot. This may be attributed, at least in part, to the very low concentrations of free Mab in each supernatant. (Table 3.4 lists the concentrations of Mab in the concentrated supernatants from the G1, G3, G4, G11, G18, G19, G24, G30 and G33 hybridomas. Related values, referred to as the concentrations of free Mab are also quoted. These are determined from the actual concentrations using the specific activity measurements tabulated in Table 3.3. The calculation of the free concentrations (see Table 3.4) is based on the assumption that the differences in specific activity of an affinity-purified Mab and its corresponding hybridoma supernatant are attributable to a reduction in the fraction of total Mab which remains free in the supernatant). The exception is the G11 Mab, whose free

concentration was very high in the supernatant used to probe the blot; the lack of staining is all the more remarkable for this. This evidence was taken as corroborating the conclusions reached with the specific activity determinations. G11 Mab was monoreactive and the remaining Mabs were polyreactive.

A final point of interest is that the differences with which the polyreactive Mabs reacted on the blot cannot be solely attributed to differences in their concentrations. G18 and G19 Mabs stained the blot with an intensity markedly higher than did the remaining Mabs even though most Mabs were used at similar concentrations to probe the blot. This difference may be a reflection of varying degrees of polyreactivity. However, it may be argued in this case that one should observe a reduction in the number of bands stained not in the intensity with which bands are stained. One other possibility is based on the suggestion, that monoclonal polyreactive antibodies might be 'organ-specific' (A. Nobrega, UFJR, Rio de Janeiro, Brasil, personal communication). Perhaps, the G18 and G19 Mabs are particularly reactive with liver proteins. To test this possibility it would be of interest to use all Mabs to probe size-fractionated proteins sourced from organs other than the liver. However, at present, the significance (if any) of this observation remains obscure.

	Concentration of Mab	Concentration of free Mab
G1	166	3
G3	106	<LOD
G4	460	61
G11	1500	1487
G18	1720	149
G19	950	6
G24	570	2
G30	96	0.06
G33	390	15

Table 3.4: *The concentrations of Mab, in each of 9 hybridoma supernatants, are tabulated above. These values were determined by standard addition as described in section 3.3.2.3. The related values for the concentrations of free Mab were calculated by multiplying the concentrations determined by standard addition by the specific activity of the supernatant; the resulting value was divided by the specific activity of the affinity purified Mab. (The relevant specific activity values are given in Table 3.3). All concentrations are related in μg/ml.*

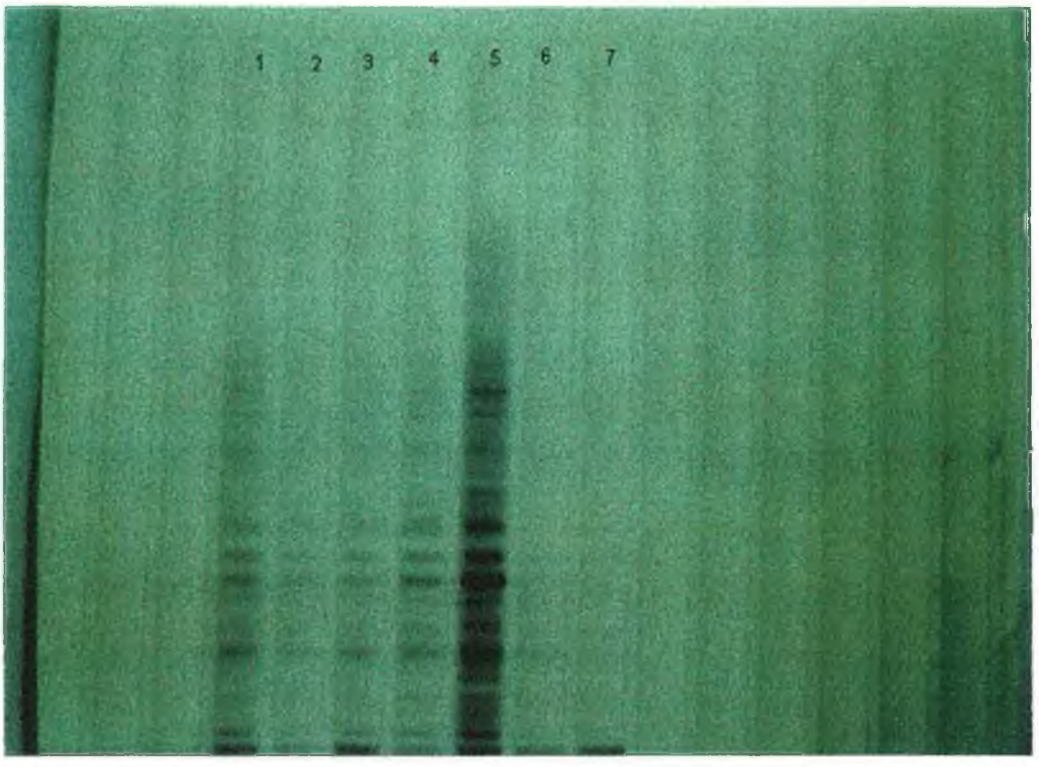


Fig. 3.63: *A liver extract (section 2.8.3.2) was run through a SDS-PAGE gel (section 2.8.3.3) and the size-fractionated proteins were electro-transferred onto nitro-cellulose (section 2.8.3.4). The nitro-cellulose was then probed with 20μg/ml solutions of the IgM fractions of G1, G4, G11, G18 and G19 supernatants (lanes 1-5, respectively) and with mouse IgM from clones 11E10 and E51.87 (lanes 6-7, respectively), as described in section 2.8.3.5.*

The results were quite surprising. Lanes 1-5 gave very similar banding-patterns, which differed only in intensity. Contrary to expectation the IgM fraction of G11 supernatant gave quite a strong banding-pattern, which indicated that it was multireactive.

Lanes 6-7, while not completely negative, were much less intense than lanes 1-5. This was as expected as these IgM fractions had been purified from two hybridomas raised by in vivo immunisation. It was, thus, expected that they would be monoreactive, generating a negligeable banding-pattern. The residual banding-patterns evident in these two lanes was attributed to the fact, that these two clones had been in the laboratory for over two years prior to use, and, hence, may have been degraded to a certain extent.

Fig. 3.64: *Liver proteins were size-fractionated and blotted onto nitro-cellulose, as described in the legend to Fig. 3.63. The nitro-cellulose was probed with G11 supernatant (conc.) containing 1,500μg/ml of G11 Mab (lane 1). It was also probed with solutions of affinity-purified G11 Mab at 36, 72, 145 or 390μg/ml (lanes 2-5, respectively). This experiment provided convincing evidence in favour of the hypothesis, that affinity-purifying the G11 Mab had converted a monoreactive antibody binding site into one, which was polyreactive. The affinity-purified G11 Mab was clearly polyreactive in that, it gave a multi-band staining pattern on the blot, even at 36μg/ml, indicating that it was reactive with many different proteins (see lane 1). In contrast, when the nitro-cellulose was probed with the G11 supernatant containing G11 Mab at the much higher concentration of 1,500μg/ml, no bands were stained, that could not be attributed to binding by the secondary antibody to the liver proteins. This indicated that the G11 Mab, in what may be described as a more natural state, was monoreactive.*

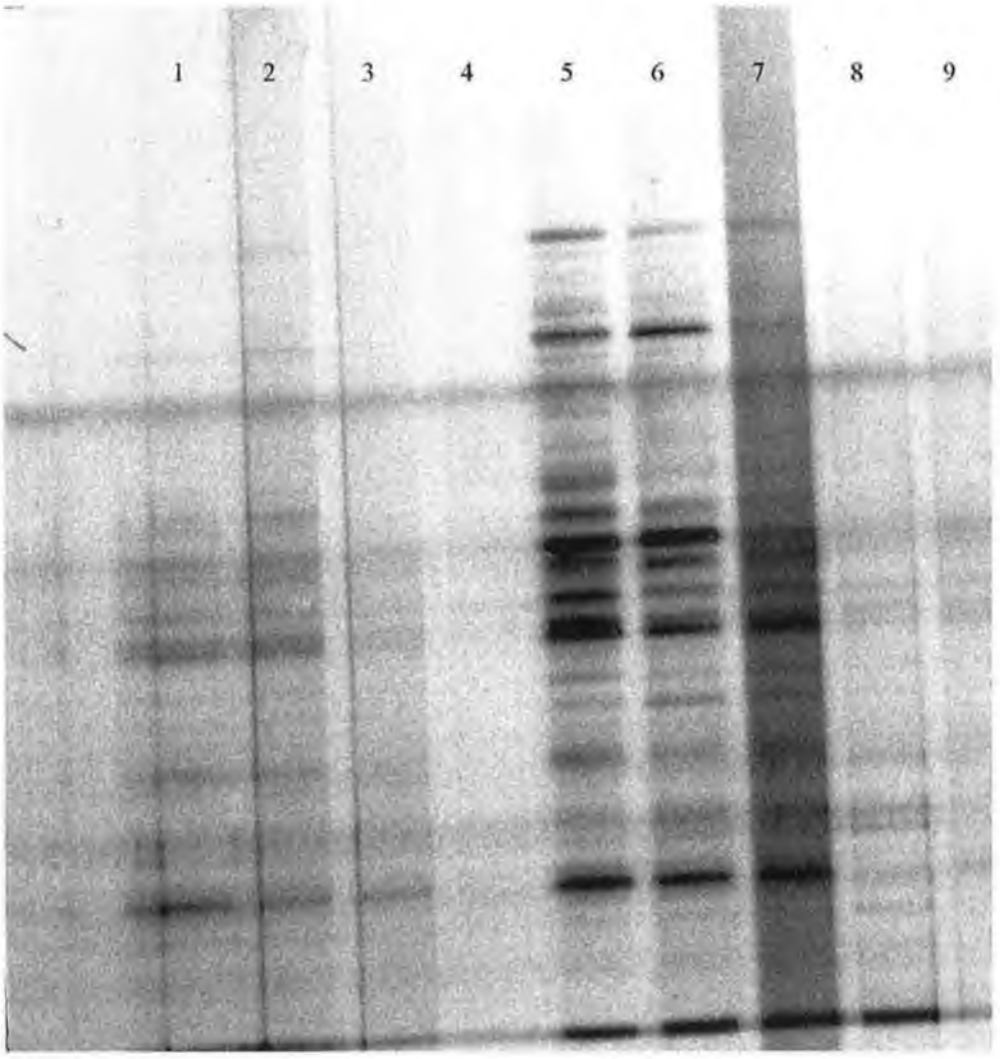


Fig. 3.65: *Liver proteins were size-fractionated and blotted onto nitro-cellulose as described in the legend to Fig. 3.63. The nitro-cellulose was probed with concentrated supernatants from the following hybridomas: G1, G4, G3, G11, G18, G19, G24, G30 and G33 (lanes 1-9, respectively). All supernatants were used neat except those from the G18 and G19 hybridomas, which were diluted 1:10 in PBS. The concentrations of Mabs in each solution can be read or calculated from Table 3.4.*

The supernatants from all hybridomas, excepting G11, gave a multi-band stain pattern. This was taken as evidence that G11 Mab was monoreactive and the remainder polyreactive.

3.4 Conclusions

It was hypothesised at the outset of this study, that IVI would produce polyreactive Mabs. To test this prediction, a panel of nine hybridomas secreting anti-GIgG (G1, G3, G4, G11, G18, G19, G24, G30 and G33) was produced using the IVI system published by Borrebaeck and Moller (1986) (section 3.2). The binding characteristics of their respective Mabs were then investigated, as described in section 3.3 (affinity determinations, specific activity determinations and immunoblots). The affinity determinations (see Table 3.2) formally identified G1, G4, G18 and G24 as polyreactive. They did not result in any classification of the remaining Mabs. In contrast, the evidence of the specific activity determinations and, also, the immunoblots (see sections 3.3.2 and 3.3.3, respectively) were in complete agreement, that 8 of the 9 Mabs produced (G1, G3, G4, G18, G19, G24, G30 and G33) were polyreactive, G11 being monoreactive. The fact that the affinity determinations only identified four of these Mabs as polyreactive is presumably due to a lack of discriminatory power, attributable to the limited number (6) of antigens involved in this experiment. Notably, both the specific activity determinations and the immunoblots involved, presumably, many hundreds of antigens (either in the supernatant or on the nitro-cellulose). Thus, these results provide explicit proof that the IVI system of Borrebaeck and Moller (1986) produces polyreactive Mabs. While these results cannot, obviously, be taken to infer that, all IVI culture systems produce polyreactive Mabs, they do at least indicate that it would be expedient to investigate this possibility for each system.

Looked at in a more general sense, these studies highlighted the problem of detecting polyreactive antibodies. The possibility that conventional assays for detecting antibody (e.g. ELISA) would not be suitable for detecting polyreactive antibodies was not considered at the start of the project. However, this appears to be the case. This point became very evident when comparing the G11 and the G30 Mabs. The reactivities of these Mabs against GIgG were indistinguishable. G30 reacted with GIgG with an affinity constant of 3.1 ($\pm$0.3) x10^{-8} while, G11 reacted with GIgG with an affinity constant of

3.5 (±0.1) $x10^{-8}$ (see Table 3.2). Nonetheless, the supernatant of the G30 hybridoma *had absolutely no anti-GIgG activity, as determined by ELISA* (see Fig. 3.21). In stark contrast, G11 supernatant *had a titre of at least 1:10,000 using this assay format* (Fig. 3.17). The explanation offered in this thesis, to account for this finding (section 3.2.1), is that when one is screening for reactivities in complex biological matrices (such as supernatants), polyreactive antibodies may be bound in solution by components of the matrix leaving only a small fraction of the antibody free in solution and, hence, detectable by ELISA. Overall, this suggests that ELISAs or any other systems for detecting antibodies in highly complex matrices are inherently biased towards the detection of monoreactive antibody. *Screens will, as a consequence, underestimate the extent of a potentially important class of antibodies. This may lead in turn to an under-appreciation of the role of polyreactive antibodies in various immune (and, if the speculations of Coutinho and Avrameas (1992) are correct, non-immune) phenomena. In order to eliminate such bias, it seems clear, that one must carry out screens in as simple a matrix as possible. Two different situations where these considerations are relevant are considered below.*

When screening cells at the clonal level (e.g. B cells or hybridoma clones), the conventional approach is to harvest the supernatant from individual clones and assay it by ELISA. The results presented in this chapter suggest that this is a sub-optimal approach, ill-suited to the detection of polyreactive antibodies. A far better approach would be to use an ELIspot assay. The coating antigen in an ELIspot assay should be better able to compete with matrix components for secreted polyreactive antibody as the ELIspot assay is based on the detection of freshly secreted antibody. *It may also be suggested that, the perfect assay for unbiased detection of both monoreactive and polyreactive antibodies would be an ELIspot assay using cells resuspended in PBS, rather than, as during these studies, culture medium.*

A different set of problems present themselves when attempting to screen complex mixtures of antibodies in an unbiased fashion. Screens of normal serum for reactivities against hundreds of antigens at once using

immunoblots (e.g. Haury *et al.*, 1994) best exemplify the problems that can occur. The problem can be stated as follows. The species of antibody present in normal serum can reasonably be expected to vary in the extent to which they show polyreactive binding profiles. Some species will be monoreactive, some highly polyreactive and others may lie somewhere between these two extremes. In a complex matrix such as serum, antibody will partition between free antibody and antibody bound to components of the serum. The problem here is that the free antibody, that is to say the portion of the total antibody detectable by immunoblot, cannot be a representative sample of the total antibody in the serum. The polyreactive antibody component of the normal serum, by its very nature, must preferentially exist bound to components of the serum; the monoreactive antibody component, by its very nature, must preferentially exist free in solution. The detectable antibody as a result must be enriched for monoreactive antibodies. The obvious solution is to affinity-purify the antibody from the serum in order to simplify the matrix in which the normal antibody is contained. Thus, Sigounas *et al.* (1994) demonstrated that normal human plasma contains much polyreactive IgM, which is masked in the circulation by antigen-binding and only revealed by affinity-purification. Berneman *et al.* (1992) showed that IgG, affinity-purified by protein G chromatography from normal mouse serum, was highly polyreactive. This polyreactivity was not evident in the serum itself.

Unfortunately, the simple expedient of affinity-purifying antibody, in order to reveal its polyreactive component, may itself lead to bias in that it may generate polyreactive antibodies from monoreactive ones. This was made very clear during the series of experiments reported in this chapter. Specifically, the G11 Mab, when exposed to low pH, was converted from a monoreactive antibody to one displaying polyreactive binding characteristics as determined by an immunoblot assay (see Fig. 3.64). That polyreactive binding evident post-affinity-purification cannot necessarily be attributed to 'de-masking' of bound polyreactive antibody, but may be artifactual and due (presumably) to limited denaturation of monoreactive antibody should obviously be borne in mind at all times. In fact, some observations in the literature, which have been attributed to 'de-masking' of polyreactive antibody may be better explained by

denaturation of monoreactive antibody. As noted in section 1.2.3, there is some dispute over the predominant isotype of natural polyreactive antibodies. Casali and Schettino (1996) have indicated that IgM is the predominant isotype. This is disputed by Avrameas and Ternynck (1995) who have claimed that IgG is the predominate isotype. There view is based on the observation that protein A-purified normal mouse IgG appears to be polyreactive, in contrast to that in the crude serum (Berneman *et al.*, 1992). This effect of purification was, similar to the ideas in this chapter, attributed to masking of reactivity in the complex biological matrix of the serum. Curiously, however, this polyreactivity was only evident upon basic elution of IgG from the protein A. Under acidic conditions, the reactivity of the IgG was not in evidence. This was claimed to be due to denaturation of the IgG under acidic conditions. While this may very well be true, one other possibility deserves consideration. This is that, the reactivity pattern is not destroyed under acidic conditions; rather *it is created under basic conditions*. In support of this notion, the conversion of G11 from a monoreactive to a polyreactive Mab is presented. It is also noteworthy that, in general, basic conditions are considered harsher on antibodies than are acidic conditions (John Quinn, D.C.U., personal communication). This interpretation of results is in agreement with other findings in the literature. For example, Klinman (1994) reported, that the repertoire of splenic B cells secreting IgG in normal mice was substantially less cross-reactive than the corresponding IgM repertoire. These observations were made with an ELIspot assay, not an ELISA, and as discussed earlier, should be able to detect polyreactive antibodies. Finally, unlike normal IgM, normal IgG production by antigen-free mice is extremely limited (Coutinho *et al.*, 1995). One possible interpretation of this is that normal IgG represents the product of a conventional immune response to external antigens. If this was the case the IgG would be expected to be monoreactive and not polyreactive.

CHAPTER FOUR

THE ANTI-NIP INTRABODY

The concept of the intrabody library and a number of its potential applications were introduced in section 1.4.4.1. These applications were considered sufficiently attractive that a project to investigate the feasibility of the intrabody library concept was undertaken and, the remainder of this thesis (this chapter and chapter 5) is dedicated to this work. The choice of this project, dependent as it is on antibody monospecificity, may seem rather incongruous when considered alongside work on antibody polyspecificity (chapter 3). This incongruity is more apparent than real, however. Polyspecific and monospecific models of antibody binding sites are not mutually exclusive, and it was felt, that as long as a sufficient fraction of the intrabody library consisted of specific intrabodies, it would prove useful.

At the outset, it was decided that, the simplest approach to generate an intrabody library, from a technological point of view, would be to convert a pre-existing phage-displayed ScFv library to an intrabody library. The only such library available at that time in the laboratory was the Nissim library (Nissim *et al.*, 1994); this library became the focus of all efforts. It was also decided at the start that, initial studies should target intrabodies to the cytoplasm.

These decisions made, a generic strategy was conceived, for converting any ScFv into an intrabody. In chapter 5, the application of this strategy to generate a 'one-shot' intrabody library is presented. In this chapter, the strategy is introduced and its validation reported.

4.1 Generic strategy for intrabody construction

The genetic construction of the Nissim library was discussed in section 1.3.3.2 and depicted in Fig. 1.8a. It contains an ORF (open reading frame) encoding a PelB - ScFv - c-myc - gIII fusion protein. When deciding how best to construct an ORF encoding an intrabody from this, the following points were noted.

i) gIII has no activity of relevance to an intrabody.

ii) In the absence of a leader sequence, proteins expressed in mammalian cells should be retained in the cytoplasm. PelB, although a bacterial leader

sequence, retains the essential feature of mammalian leader sequences, that is to say, it contains a core of hydrophobic amino acids. Therefore, the possibility existed that a PelB - ScFv - c-myc fusion protein would be targeted to the ER of a mammalian cell prior to secretion.

These considerations suggested the ScFv - c-myc fusion protein as an appropriate intrabody format. While the c-myc tag, like gIII, has no essential activity, it was retained for the purpose of analysing the intracellular location of the putative intrabody. (A protein G affinity-purified anti-c-myc was available in the laboratory. It had been purified from the supernatant of the Mycl-9E10 hybridoma).

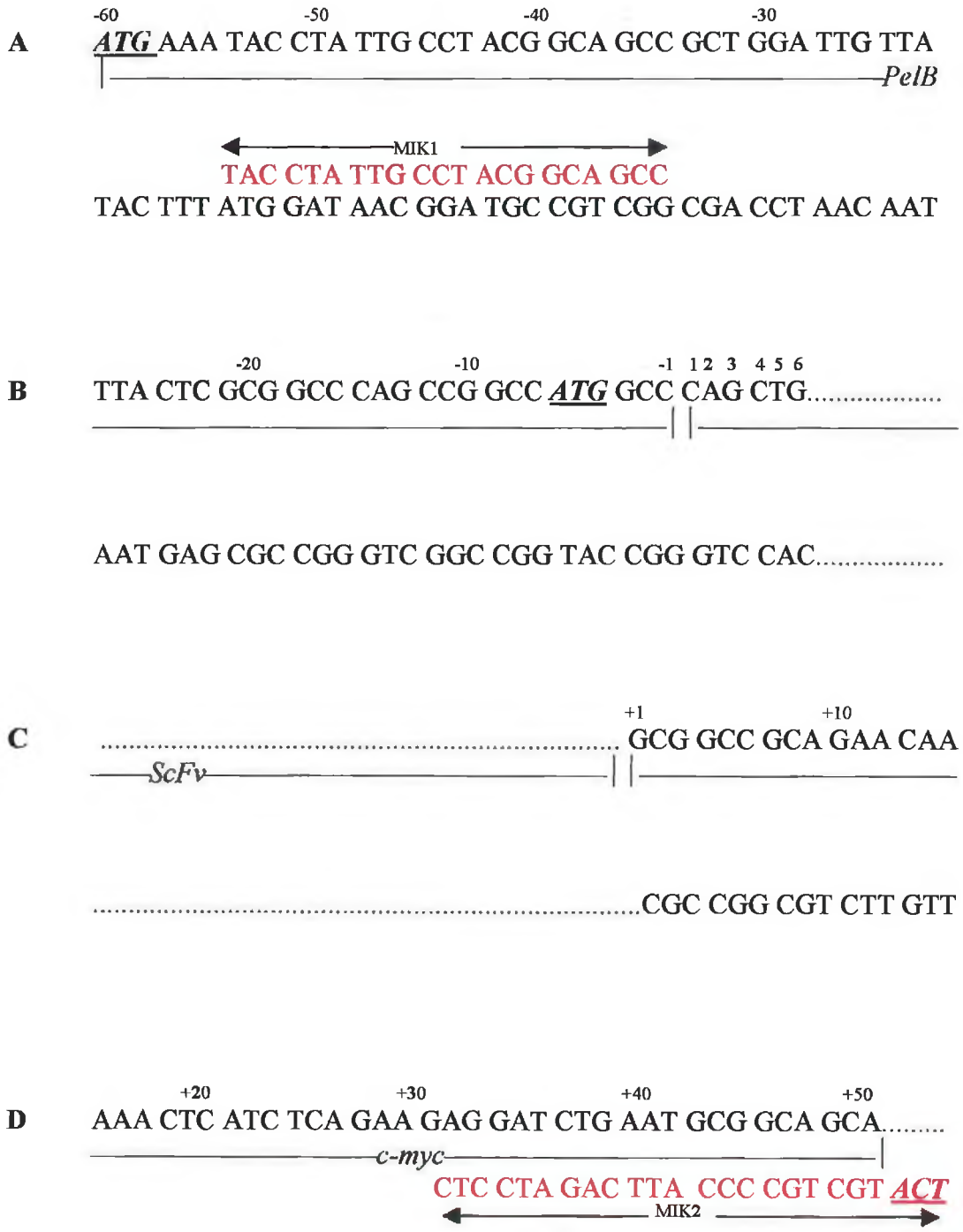
PCR was the obvious strategy to generate an ORF encoding a ScFv - c-myc protein from that encoding a PelB - ScFv - c-myc - gIII protein. The insertion of a stop codon, just 3' to the *c-myc* sequence could be achieved easily using a primer referred to as MIK2 (see Fig. 4.1). Of great importance for the aim of generating an intrabody library, this primer could be used to construct an intrabody from any ScFv in the Nissim library, or indeed, an intrabody library from the Nissim library. This was due, simply, to the fact that the sequence this primer targeted was part of the parental plasmid used to construct the Nissim library (see section 1.3.3.2) and was present on every member of the library.

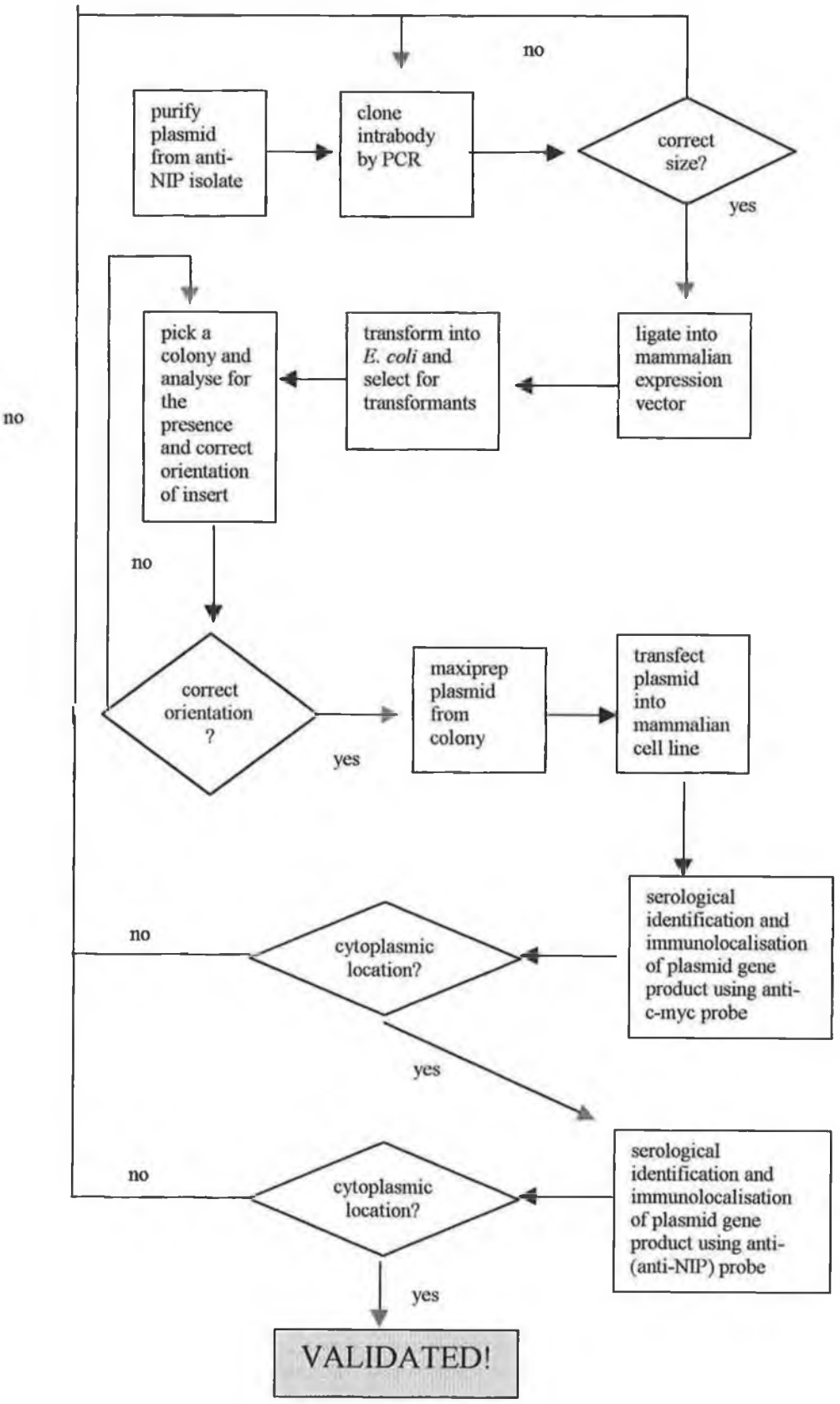
The insertion of a start codon just 5' to the *ScFv* sequence was a more formidable task. There were two conflicting requirements. On the one hand, it was desired to target the forward primer within the *PelB* sequence (which is encoded on the parental plasmid) so that the primer would be generally applicable. On the other hand, there was no obvious way to introduce the required start codon, using a primer targeted to this region.

Fortunately, a closer look at the detailed sequence of *PelB* revealed, that as well as the initiator ATG codon at its 5' end, it contained an 'in-frame' ATG codon at its 3' end (Fig. 4.1). This codon could be used to start the ORF encoding the ScFv - c-myc protein. Thus, the forward primer could be targeted anywhere between the two ATG codons. All potential primers were

analysed using GeneStrider™ for the potential to form hairpin bends and loops. Based on this analysis, the primer named MIK1 was chosen.

Prior to applying this strategy to create an intrabody library, it was validated using an anti-NIP ScFv (4-hydroxy-5-iodo-3-nitrophenylacetyl) isolate provided with the Nissim library. The remainder of this chapter is concerned with the validation of this strategy (Fig. 4.2).


A
-60
-50
-40
-30
ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA
PelB
MIK1
TAC CTA TTG CCT ACG GCA GCC
TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT
B
-20
-10
-1 1 2 3 4 5 6
TTA CTC GCG GCC CAG CCG GCC ATG GCC CAG CTG.....
AAT GAG CGC CGG GTC GGC CGG TAC CGG GTC CAC.....
C
+1
+10
..... GCG GCC GCA GAA CAA
ScFv
.....CGC CGG CGT CTT GTT
D
+20
+30
+40
+50
AAA CTC ATC TCA GAA GAG GAT CTG AAT GCG GCA GCA.....
c-myc
CTC CTA GAC TTA CCC CGT CGT ACT
MIK2
TTT GAG TAG AGT CTT CTC CTA GAC TTA CCC CGT CGT.....


purify plasmid from anti-NIP isolate
clone intrabody by PCR
correct size?
no
yes
ligate into mammalian expression vector
transform into *E. coli* and select for transformants
pick a colony and analyse for the presence and correct orientation of insert
no
no
correct orientation ?
yes
maxiprep plasmid from colony
transfect plasmid into mammalian cell line
serological identification and immunolocalisation of plasmid gene product using anti-c-myc probe
no
cytoplasmic location?
yes
serological identification and immunolocalisation of plasmid gene product using anti-(anti-NIP) probe
no
cytoplasmic location?
yes
VALIDATED!

4.1.1 Polymerase chain reaction: Plasmid DNA from the anti-NIP ScFv isolate was prepared as described in section 2.7.2.1 (Fig. 4.3), restricted with *Eco*RI and gel-purified according to section 2.7.2.1. This was used as the template DNA in a PCR, as described in section 2.7.3.1. MIK1 and MIK2 were used as the primers. The PCR was analysed by gel electrophoresis, as depicted in Fig. 4.4, and was very successful, as described in the accompanying legend.

4.1.2 'TA Cloning' of the PCR product and transformation of the resulting recombinant plasmid into *E. coli* Top10F': Due to a nontemplate-dependent polymerase activity, PCR products produced using *Taq* polymerase contain 3' adenosine overhangs. As a result, they can be ligated into linearised vectors containing complementary 3' thymidine overhangs, a process known as 'TA-cloning' (Mead *et al.*, 1991; Hengen, 1995). Although not the most efficient way to clone PCR products, its simplicity (it does not require purification of the PCR product) makes it the most attractive methodology when its limited efficiency is not an issue.

A commercially available mammalian expression vector, pCR™3-Uni (see appendix D for plasmid map and sequence), was used for TA cloning. It came as part of the 'Unidirectional TA Cloning Kit', which also provides for transformation of the resulting ligation mix into *E. coli* Top10F'. In total, 120ng of PCR product was ligated with 60ng of pCR3™-Uni, and 36ng of the resulting product was transformed into *E. coli* Top10F', as instructed by the manufacturer's, resulting in 350 transformants.

Unfortunately, this system does not allow for selection of transformants containing recombinant over non-recombinant plasmids. Nor does it allow selection of recombinants containing insert in the correct orientation. Thus, after growth of the transformed bacteria, individual colonies must be screened for the presence of a correctly oriented insert.

Fig. 4.3

Fig. 4.4

Fig. 4.3: *Plasmid (referred to as pHen-V_λ + anti-NIP ScFv) was purified from an anti-NIP ScFv isolate provided with the Nissim library. It was restricted with EcoRI and size-fractionated by gel electrophoresis to estimate its size.*

Lanes 1 and 6: M_w markers as indicated in the margin (Lambda DNA restricted to completion with EcoRI and HindIII).

Lanes 2 and 3: Plasmid DNA from the anti-NIP ScFv isolate, before (2) and after (3) restriction with EcoRI.

Lanes 4 and 5: Parent plasmid (pHen1) before (5) and after (4) restriction with EcoRI.

Note that, when restricted, the plasmid from the anti-NIP ScFv isolate appeared at ≈ 5,300bp, as one would expect (pHen1 was 4,500bp and the insert was expected to be 780bp in size). The restricted pHen-V_λ + anti-NIP ScFv was subsequently eluted from the gel and used as the template DNA in a PCR, to create an ORF encoding an intrabody

Fig. 4.4: *A PCR was set up as described in section 2.7.3.1. Restricted, gel-purified pHen-V_λ + anti-NIP ScFv was used as the template DNA. MIK1 and MIK2 were used as forward and reverse primers, respectively. The success of the PCR was assessed by gel electrophoresis.*

Lanes 1 and 7: M_w markers as indicated in the margin.

Lanes 2-6: PCR product.

The PCR appeared to work very well. Only one band was evident and it appeared at 780bp as expected.

Screening was carried out, by purifying the plasmid from individual colonies (section 2.7.2.1) and restricting it with *Nco*I. There was a *Nco*I site at the 5' end of the PCR product (see Fig. 4.1, positions -8 to -3). This combined with the fact that, pCR3™-Uni contains three *Nco*I sites oriented asymmetrically about its multiple cloning site, allowed one to identify recombinant plasmids with the insert in the correct orientation, based on the *Nco*I restriction pattern (Fig. 4.5).

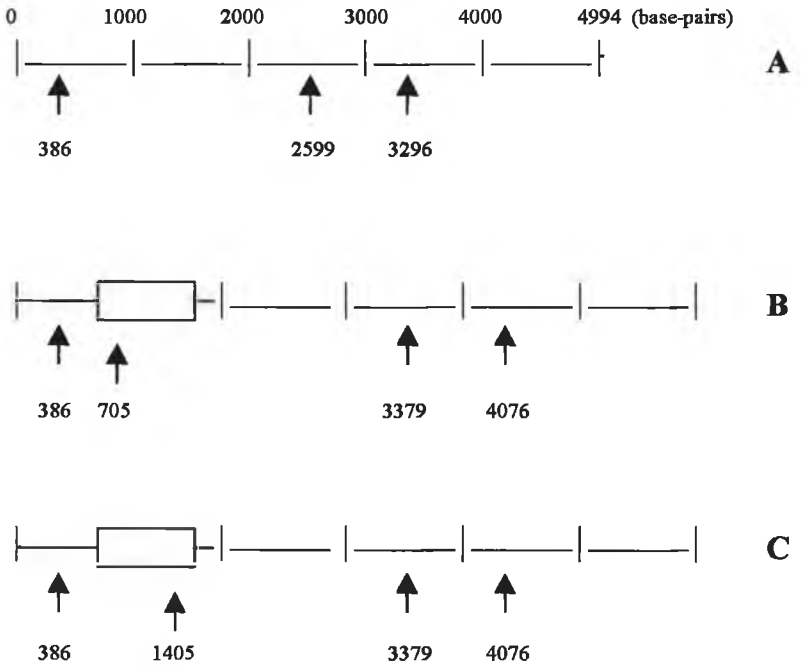


Fig. 4.5: *A, B and C represent schematics of pCR3™-Uni without insert, with insert in the correct orientation and insert in the incorrect orientation, respectively. Arrows indicate NcoI sites. A NcoI restriction pattern composed of 4 bands of 339, 2674, 697 and 2187 base-pairs is indicative of a correctly oriented insert. These calculations are based on an insert of 780 base-pairs.*

Seven colonies were analysed for the presence of a correctly oriented insert and the results are shown in Fig. 4.6. A clone referred to as Top6 contained a correctly oriented insert. This plasmid was named pp6. In the next section, studies aimed at determining the sub-cellular location of its gene product are described.

Fig. 4.6: *Plasmids were purified from 7 transformants (Top2-Top8) according to section 2.7.2.1 and, subsequently, restricted with NcoI. The restriction fragments were size-fractionated by gel electrophoresis.*

Lanes 1, 6 and 10: M_w markers as indicated in the margin.

Lanes 2-5 and 7-9: Plasmids from colonies Top2-Top8, restricted with NcoI.

Note that, the plasmids from Top3 and Top6 (referred to as pp3 and pp6, respectively) appeared to contain the insert in the correct orientation, as indicated by the presence of a band at ≈ 340bp.

4.2 Transfection experiments

In order to both serologically identify, and locate at the sub-cellular level, the gene product expressed by pp6, it was transfected into three different cell lines, NIH3T3, CHO and BHK, chosen for their high transfectability. After 48 hrs, the transiently transfected cell populations were analysed by immunocytochemistry. Alternatively, stably transfected cell lines were selected and analysed. Stable transfectants lend themselves to immunocytochemical analyses using electron microscopy, which was expected to lead to a deeper appreciation of the sub-cellular localisation of the gene product.

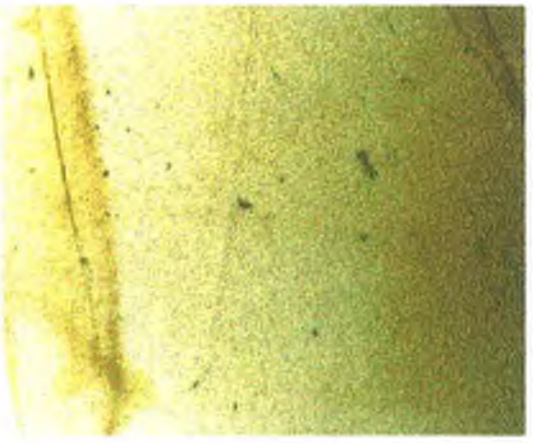
Two different probes were used during the immunocytochemical analyses including the anti-c-myc probe mentioned in section 4.1. An anti-NIP antiserum produced in a rabbit during the course of these studies, was also used.

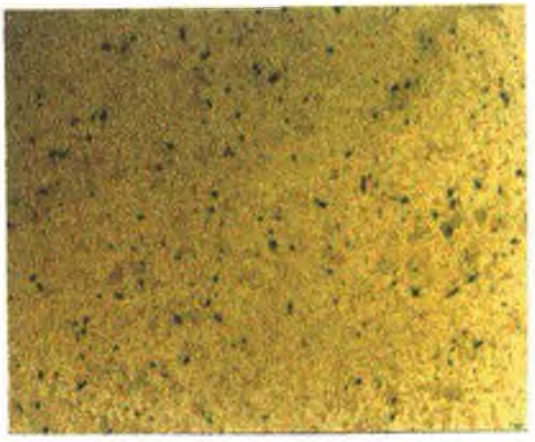
4.2.1 Optimisation of transfection conditions: Calcium phosphate transfection, DEAE-dextran transfection, electroporation and lipofection are the four major methods for introducing DNA into mammalian cells, of which, lipofection is the most efficient (Ausubel *et al.*, 1990). A number of lipofection reagents are available commercially, and the Tfx™ series (Tfx™-10, 20 and 50) was chosen at random for these studies. It is recommended that transfection conditions be optimised for each individual cell line. Parameters to be optimised include the type of Tfx™ reagent used, the DNA concentration and the charge ratio of Tfx™ : DNA in the transfection mix, and the lenght of time that it is applied to the cells.

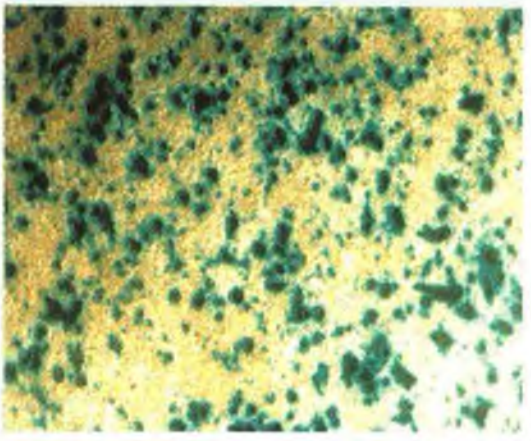
In order to optimise these parameters, pCH110 was transfected into the cell lines under the variety of conditions suggested in Promega Technical Bulletin # TB 216 (DNA concentrations of 5, 2.5, 1.25 or 0.625μg/ml; charge ratio's of 2:1, 3:1 and 4:1; time periods of 1 or 2 hrs.). pCH110 is a reporter plasmid expressing β-galactosidase (see appendices B and D), and the effectiveness with which it was transfected was monitored by analysing the percentage of cells in the transfected population expressing this enzyme (see section 2.8.2.1). The optimal transfection conditions are shown in Table 4.1.

	Tfx™	DNA (µg/ml)	Tfx™:DNA	Time (hours)
NIH3T3	50	2.5	3:1	1
CHO	50	5	4:1	2
BHK	10	2.5	2:1	1

Table 4.1: *The optimised transfection conditions for the three cell lines used for this study are shown above. The transfection efficiency for each cell line (% cells expressing β-galactosidase) is visually depicted in Figs. 4.7-4.9.*







Figs. 4.7-4.9 (clockwise from top left): *NIH3T3, CHO and BHK cells (Figs. 4.7-4.9, respectively), were transfected with pCH110 as described in section 2.5.8.1 and under the conditions noted in Table 4.1. After 48hrs the cells were monitored for β-galactosidase expression as described in section 2.8.2.1. 0.1%, 1% and 10%-30% of NIH3T3, CHO and BHK cells, respectively, appeared to express β-galactosidase. (40x magnification).*

4.2.2 Immunocytochemical analysis of the pp6 gene product using the anti-c-myc probe: A maxiprep of pp6 was prepared as described in section 2.7.2.2. It was transfected into the NIH3T3 cell line, as described in section 2.5.8.1, under optimised conditions. The resulting cell population was analysed with the anti-c-myc probe as described in section 2.8.2.2. A photograph of a typical stained cell is presented in Fig. 4.10. The cellular distribution appeared diffuse and was not associated with any particular cellular structure. This is what one would expect for a cytosolic protein. In particular, there was no clear-cut exclusion from the nucleus; this again is what one would expect for a cytosolic protein of 31kDa (Biocca *et al.*, 1990). Molecules smaller than 40kDa appear able to diffuse into the nucleus through the nuclear pores (Biocca and Cattaneo, 1997). It was also noted that, in so far as one could tell visually, the expression level of the gene product appeared to be quite good[1] (appendix B).

Unfortunately, when the CHO and BHK cell lines were similarly analysed the results were very disappointing. CHO cells transiently transfected with pp6 did not show any indication of gene product expression as detected by anti-c-myc. While, some BHK cells did appear to express the pp6 gene product in the cytosol (see Fig. 4.11), expression levels appeared to be quite poor. Only $\approx$ 0.05% of transfected BHK cells appeared to be expressing the gene product. This compared very unfavourably with the 10-30% of cells expressing β-galactosidase from pCH110 (see appendix B). This difference was attributed to low expression of the pp6 gene product compared with that of pCH110. (It could not be attributed to a toxic effect of the pp6 gene product as, when a mixture of pp6 and pCH110 was transfected into BHK cells, no diminution in β-galactosidase expression was noted (result not shown)).

Overall, these results suggested that the pp6 gene product was expressed in the cytoplasm but at low levels. In order to confirm this sub-cellular location, stably transfected NIH3T3 clones were selected with 800μg/ml of geneticin as described in section 2.5.8.2. A typical outgrowing colony is depicted in Fig. 4.12.

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[1] The maximum number of cells per smear was 50, 000 (50μl of a $1x10^6$ cell/ml cell suspension was cytospun onto a glass slide) and in general 10 cells per smear were fluorescent. As a percentage this is 0.02%. This compared relatively favourably with the 0.1% transfection efficiency observed for this cell line with the pCH110 reporter plasmid.

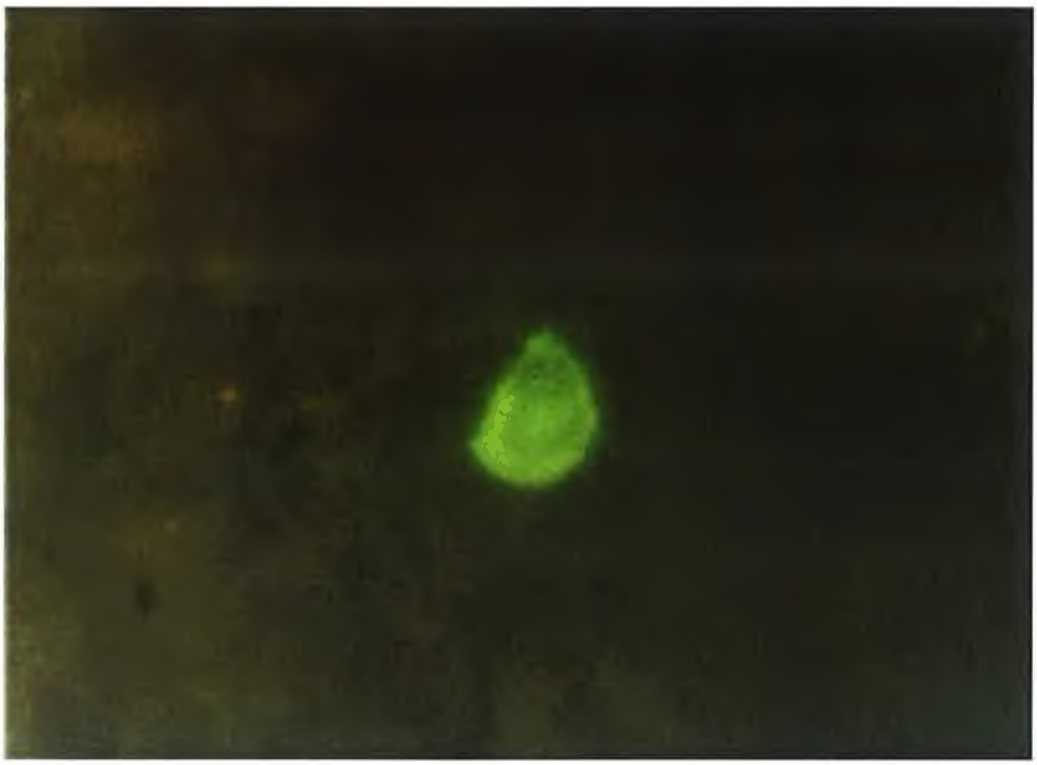


Fig. 4.10: *An NIH3T3 cell expressing pp6 gene product is presented. The product was detected as described in section 2.8.2.2. The anti-c-myc primary probe was used at a dilution of 1:50, which in a preliminary experiment was the highest concentration of probe that could be used without excessive background non-specific staining. This probe was detected with a FITC-anti-mouse IgG probe used at the manufacturer's recommended dilution. Note the diffuse staining throughout the entire cell, which is less intense throughout the nucleus. This staining pattern and, particularly, the lack of exclusion from the nucleus is what one would expect of a small cytosolic protein. (1000x magnification).*

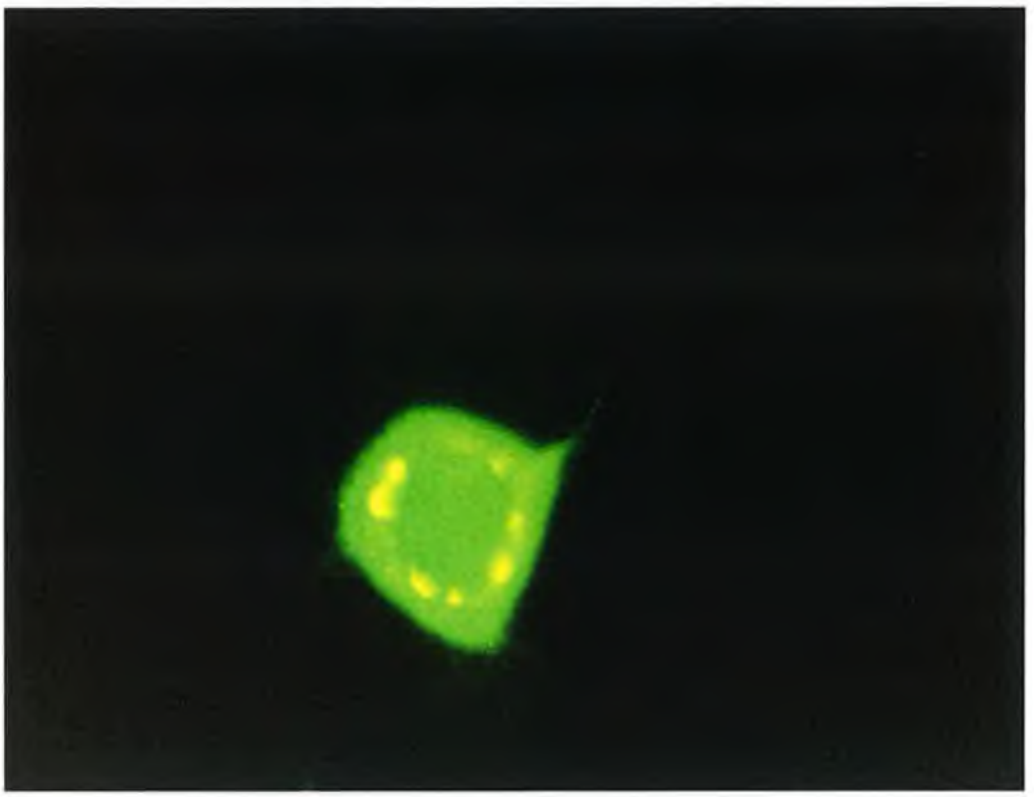


Fig. 4.11: *A BHK cell expressing pp6 gene product is presented. The gene product was detected as described in the legend to Fig. 4.10 and the distribution of the product was very similar to that of Fig. 4.10. (1000x magnification).*

The efficiency with which cells transfected with the Tfx™ reagents become stably transfected has been shown to be remarkably high. Schenborn *et al.* (1995) reported that, 17% of NIH3T3 cells were transiently transfected by Tfx™-50 and 5% of the cells were stably transfected. Thus, ≈ 30% of cells taking up DNA become stably transfected. The results reported here are in agreement with this. In one experiment, $1.5x10^6$ cells were transfected with pp6 and 48 hrs later were split 1:15 into 60mm tissue culture dishes in order to select for stable transformants. After 10 days, one dish was Giemsa stained in order to count the number of colonies on it (Fig. 4.13). Approximately, 50 colonies were obtained suggesting that, 750 stable transformants had resulted. This was 50% of the number of cells taking up DNA in the first instance (asssuming a transfection efficiency for NIH3T3 cells of 0.1%).

In any case, 19 colonies were picked and established (see Fig. 4.14) as cell lines. They were named N1 - N19. To ensure that the cell lines were true stable transfectants, genomic DNA was prepared (section 2.7.2.3 ii) from a random sample of these cell lines (N8, 9, 10 and 12) and also from the parental NIH3T3 cell line. Each of these 5 samples was interrogated, by PCR, for integration of pp6. The genome preparations were used as template DNA, along with the MIK1 and MIK2 primers, in a PCR as described in section 2.7.3.1. If the genomic DNA contained a copy(s) of pp6, the primers would be expected to amplify its 780bp ORF. The result of this experiment is contained in Fig. 4.15. Quite clearly, all sub-clones gave rise to a 780bp PCR product. Just as importantly, the parental cell line did not. This established that, these 4 particular sub-clones were genuine stable transfectants. It also suggested that, the entire panel represented stable transfectants. A 650bp band is evident in all 5 PCRs, presumably representing the product of amplification of a sequence carried on the parental genome.

When these cell lines were analysed for expression of the pp6 gene product, by immunocytochemistry with the anti-c-myc probe, all were negative. This suggested that, the gene product was expressed at very low levels and, as a result, probably ruled out the application of electron microscopy techniques to further probe its sub-cellular distribution.

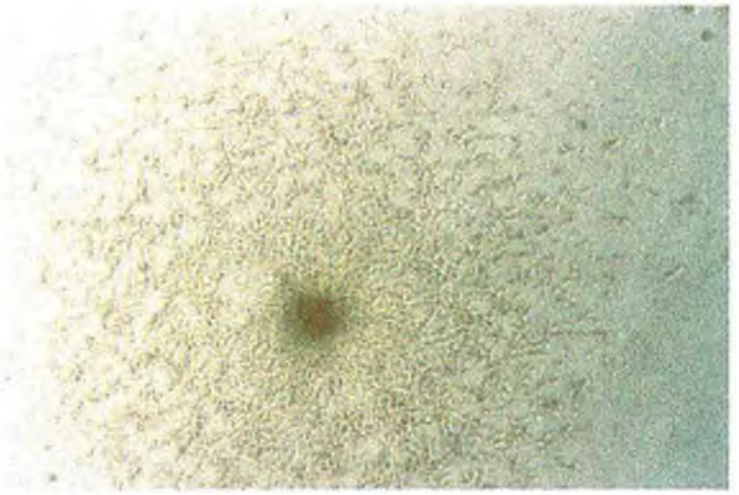


Fig. 4.12: *A colony of NIH3T3 cells selected with 0.8mg/ml of geneticin, is presented in the photograph (40x magnification). The cells towards the centre of the colony appeared to be dead. Colonies, in general, were picked when they had reached this size, and were treated as stably transfected clones. The scale-up procedure used to establish colonies as cell lines is given in Fig. 4.14.*

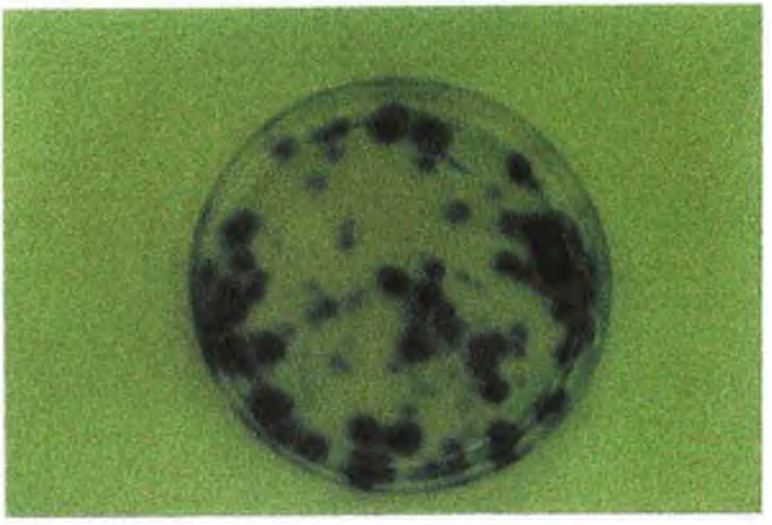


Fig. 4.13: *A Giemsa stained 60mm tissue culture dish containing NIH3T3 colonies selected with geneticin is depicted. The dish was stained as follows. The medium was poured-off the cells, and the cell monolayer was washed with PBS. The cells were fixed for 15 minutes with ice-cold methanol. The fixed cells were stained with a 10% (w/v) solution of Giemsa in methanol, for 15 minutes at RT, prior to rinsing the dish with tap-water. The above dish contained approximately 50 colonies.*

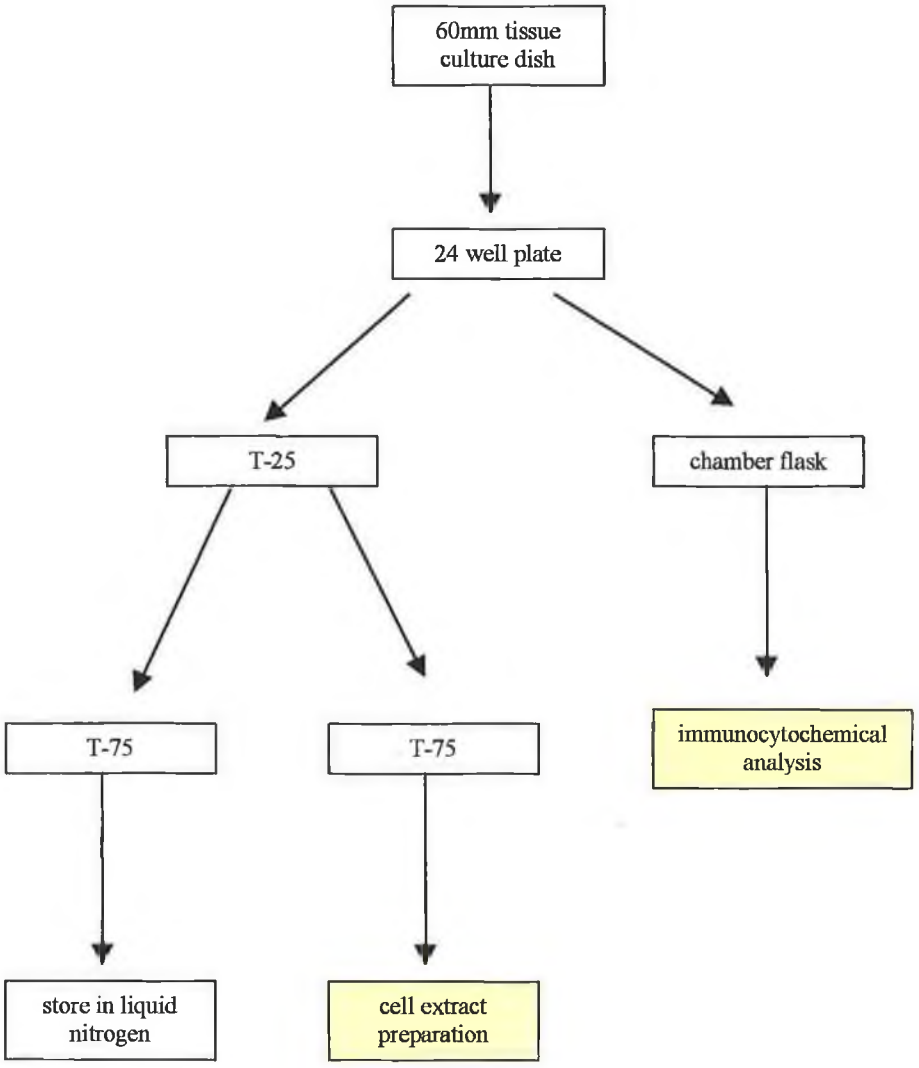


Fig. 4.14: *The scale-up procedure used to establish stable transfectant colonies as cell lines is presented. Note that, the colonies were screened by immunocytochemistry for expression of the pp6 gene product as soon as was practically possible. Also, cell extracts were prepared as described in section 2.8.3.1. Immunoblot analysis of the cell extracts (see section 4.2.3.3) was alternative to and independent of immunocytochemistry as a means of monitoring the presence of pp6 gene product.*

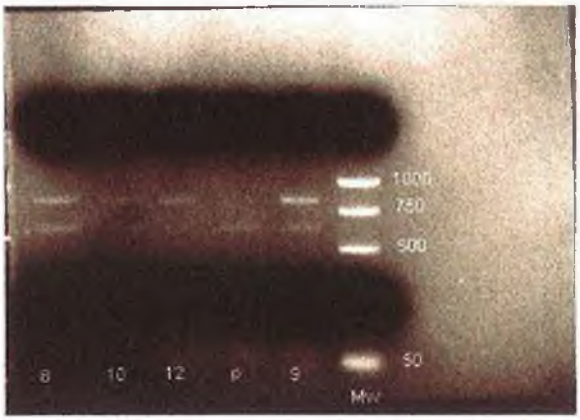


Fig. 4.15: *In order to determine whether N8, N9, N10 and N12 contained pp6 integrated into their genome, their genomic DNA (along with that from the parental NIH3T3 cell line (p)) was purified (section 2.7.2.3). This DNA was amplified with the MIK1 and MIK2 primers, essentially as described in section 2.7.2.1. The PCR product obtained was size-fractionated by gel electrophoresis (product obtained using N8 was run through the gel in the lane indicated 8 etc.). Notably, all four sub-clones contained a 780bp amplification product, which is what one would expect if they contained pp6 integrated into their genome. The parental cell line does not show this amplification product.*

Overall, the results obtained with the anti-c-myc probe were quite disappointing. The pp6 gene product did appear to be expressed in the cytoplasm, but electron microscopy studies to confirm this could not be carried out due to the poor expression levels observed. Although studies to analyse the rates of transcription and translation of this ORF were not carried out, the reason for this poor expression is almost certainly due to rapid degradation of the protein. This assertion is made as pp6 contains a very active promoter (appendix B and D) (therefore, it is reasonable to expect high rates of transcription) and, as pointed out in Fig. 4.1, the ORFs start codon occurs in the context of a consensus Kozak sequence (therefore, one would expect effective translation of its mRNA). Rapid degradation of cytosolic intrabodies has been noted by Biocca *et al.* (1995); these authors attributed this to poor folding of the variable domain in the reducing environment of the cytoplasm. Degradation is probably exacerbated by the intrabody format chosen for these studies. The c-myc protein (part of which was chosen to be fused to the ScFv, for no better reason than aiding detection of the gene product) is a very short-lived protein with an intracellular half-life of only 0.5 hrs (Dice, 1987). It is very possible that the c-myc tag confers this trait on the pp6 gene product.

4.2.3 Immunocytochemical analysis of the pp6 gene product using an anti-NIP ScFv antiserum: An anti-NIP ScFv antiserum was raised in a rabbit for use as a immunocytochemical probe for the pp6 gene product. This was important for three reasons. Firstly, as the pp6 insert had not been sequenced, it was important to identify that the gene product did indeed possess, as designed, the anti-NIP ScFv moiety. Secondly, the possibility that the c-myc tag was being rapidly *but selectively* degraded *in vivo* was considered. This was possible, as such observations had been made *in vitro* (T. Killard, DCU, personal communication). Such selective degradation would leave the ScFv intact, but undetectable with the anti-c-myc probe and was considered a possible explanation for the poor expression levels observed with this probe. Finally, the presence of such an antiserum would allow the design of the intrabody without the c-myc tag, potentially improving its *in vivo* stability.

4.2.3.1 Purification of the anti-NIP ScFv: The anti-NIP ScFv isolate from the Nissim library (referred to as *E. coli* HB2151 anti-NIP ScFv) was cultured under such conditions as to express the anti-NIP ScFv (see section 2.6.4). The culture broth (500ml) and a periplasmic lysate (10ml) were prepared from the culture supernatant and corresponding cell pellet, respectively. The anti-NIP ScFv activity, of the solutions, was assessed by ELISA using a BSA-NIP conjugate as coating antigen (kindly provided by T. Killard, DCU) (Fig. 4.16).

The ScFv was affinity-purified using BSA-NIP immobilised on Sepharose 4B. This solid phase was prepared essentially as described in section 3.3.2.2. The only difference was that BSA-NIP was used rather than anti-μ. It was used at a concentration of 10mg/ml in a total volume of 10ml of coupling buffer. This immobilisation was more efficient than that with anti-μ. The spectrophotometric analysis revealed that > 98% of the BSA-NIP was immobilised giving a ligand density of 10mg protein per ml of wet gel. 2ml of the wet gel was packed in a column and used to purify the anti-NIP ScFv.

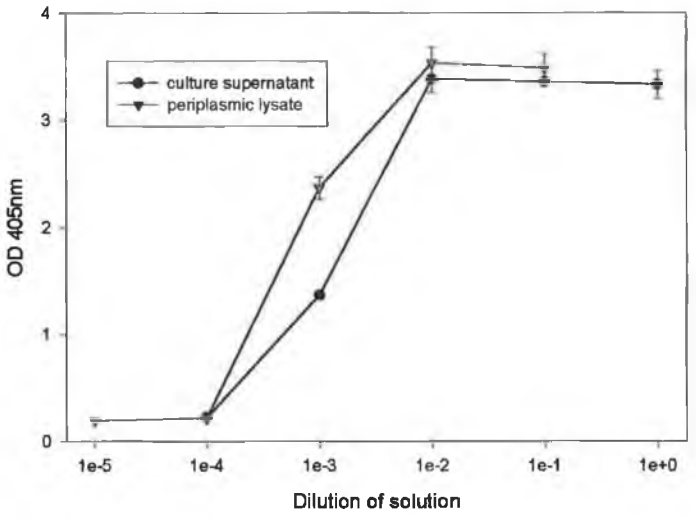


Fig. 4.16: *A culture broth and a periplasmic lysate were prepared from E. coli HB2151 anti-NIP ScFv (section 2.6.4) as a source of anti-NIP ScFv. The anti-NIP ScFv activities of both solutions were titrated by ELISA. Both the culture supernatant and the periplasmic lysate appeared to contain a large amount of the ScFv, as indicated by the presented titration curves. Although the periplasmic lysate appeared to have a slightly greater concentration, the culture supernatant was present in greater volume and was used as the starting source for the purification of the ScFv.*

ELISA: The ELISA was carried out in a similar fashion to section 2.8.1.1. A 10μg/ml solution of a BSA-NIP conjugate was used as the coating antigen. The test solutions were incubated in the coated and blocked wells as described in section 2.8.1.1. A two-step process was used to detect bound ScFv. Firstly, a protein G-purified mouse IgG_1 monoclonal anti-c-myc was allowed to react with the bound ScFv (1:1000 dilution of the monoclonal antibody, in PBS containing 0.05% (v/v) Tween and 5% (v/v) FCS, was incubated in the wells for 1 hr.). The bound mouse IgG_1 antibody was, subsequently, detected with alkaline phosphatase conjugated to goat anti-mouse IgG_1, followed by substrate, as described in section 2.8.1.1.

200ml of the culture supernatant (referred to as 'fresh supernatant') was passed through the column (and collected - referred to as 'spent supernatant'), followed by 30ml of PBS. Optimal elution conditions were not known and the initial attempt at elution used 0.1M glycine, pH 2.4. 1ml fractions were collected, neutralised with 0.1ml of 1M Tris.Cl, pH 8.7, and the presence of protein analysed spectrophotometrically (OD 280nm). The elution profile is shown in Fig. 4.17.

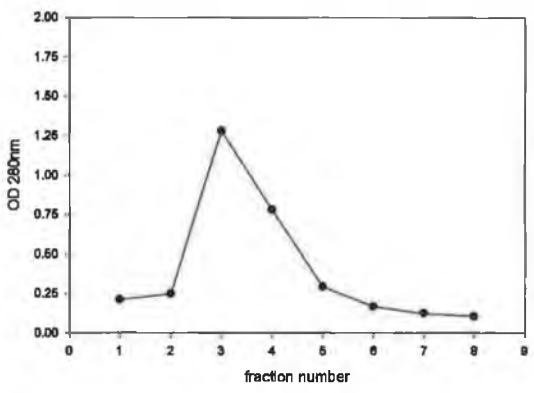


Fig. 4.17: *200ml of culture supernatant containing anti-NIP ScFv was passed through a BSA-NIP column. The retained protein was eluted off the column using 0.1M glycine, pH 2.4, collected in 1ml fractions and neutralised. The quantity of protein in each fraction was determined by reading their optical densities at 280nm. The resulting elution profile indicated that one relatively sharp peak eluted off the BSA-NIP column, presumably representing anti-NIP ScFv. Fractions 3 and 4 were pooled, dialysed overnight against PBS containing 0.5M NaCl and stored at 4^0C. The anti-NIP ScFv activity of this fraction was analysed, subsequently, as was its purity.*

The success of the affinity purification was first analysed by evaluating the anti-NIP ScFv activity of the fresh and spent supernatant and the affinity-purified fraction (Fig. 4.18). Two points are clear from this graph. Firstly, the column was very efficient in that it removed nearly all the anti-NIP ScFv activity from the culture supernatant (compare the fresh supernatant with the spent supernatant). The second point to note is that, the affinity-purified sample had 50 times the activity of the fresh supernatant. Now, if the affinity purification had been 100% successful (100% recovery of anti-NIP ScFv with full retention of specific activity) one would have expected a 100-fold increase

in activity (200ml fresh supernatant, only 2ml of affinity-purified sample). Although the recovery of protein was certainly not 100%, it was probably over 90% given the sharp peak that eluted off the column. This suggested that the affinity purification lead to a 50% decrease in specific activity. The reason for this is not readily apparent. Certainly, the elution pH used was quite gentle. One possibility is that, some of the anti-NIP ScFv in the fresh supernatant existed in dimerised form (Nissim *et al.*, 1994) and had a higher specific activity than the monomeric form due to the 'bonus effect of multivalency'. Upon exposure to low pH it is reasonable to suggest that these dimers may have been converted back to monomers - explaining the reduced specific activity of the affinity-purified fraction compared to the fresh supernatant.

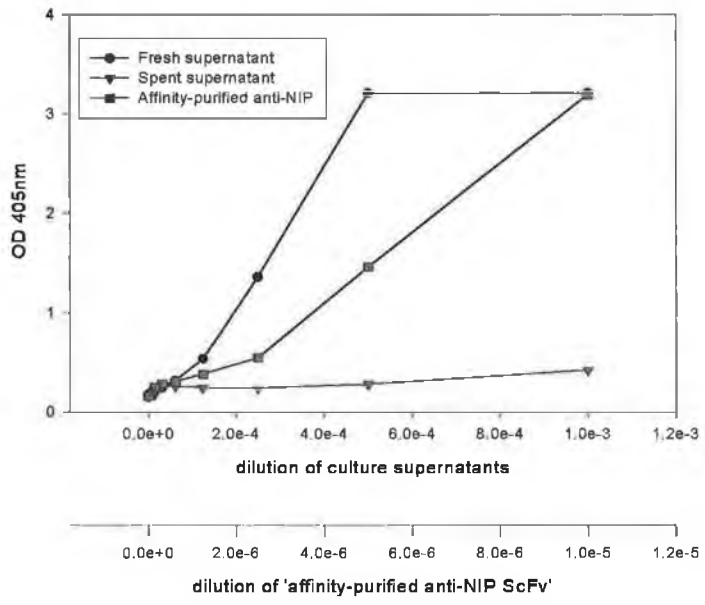


Fig. 4.18: *The anti-NIP ScFv activity possessed by a supernatant, both before ("fresh") and after ("spent") being passed through a BSA-NIP column, along with that possessed by the eluted protein ("affinity-purified anti-NIP ScFv") was titrated by ELISA (carried out as described in the legend to Fig. 4.16). The data suggests that, the affinity-purified sample contained 50-times more anti-NIP ScFv activity than the fresh supernatant.*

Finally, the purity of the affinity-purified fraction was assessed by SDS-PAGE analysis (section 2.8.3.3). The SDS-PAGE gel was stained with Coomassie blue and the resulting banding pattern is presented in Fig. 4.19.

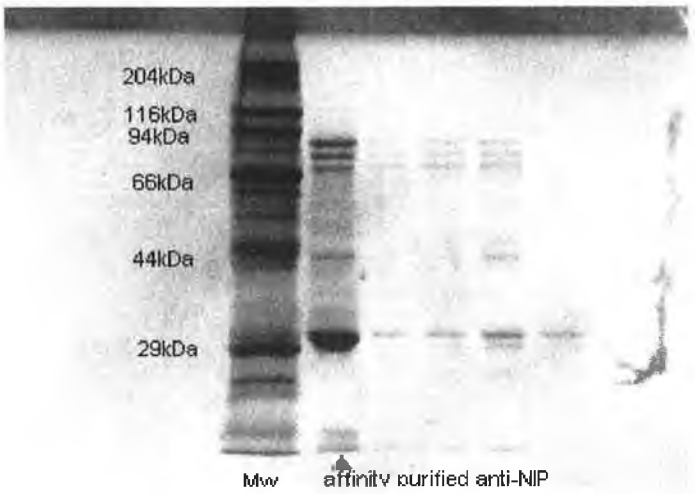


Fig. 4.19: *The affinity-purified anti-NIP ScFv was run through a SDS-PAGE gel and stained with Coomassie Blue, as described in section 2.8.3.3. Although one major band was evident at 31kDa, and was tentatively identified as the anti-NIP ScFv, many other contaminating proteins were present in this preparation. (Fractions containing anti-NIP ScFv, affinity-purified from the periplasmic lysate, were run in the unlabelled lanes).*

The result was quite disappointing. As expected one major band occurred at 31kDa, the expected size of a ScFv. However, many other higher molecular weight bands were evident with one or two minor low molecular weight bands. A rough assessment suggested that, the affinity-purified fraction was composed of only 50% (w/w) ScFv - the remainder being composed of contaminating proteins.

Although not ideal for *in vivo* immunisation, it was felt that, overall, it was not worthwhile investing time and effort to further improve the purity; accordingly, this fraction was used as the immunogen to generate the anti-NIP ScFv antiserum.

4.2.3.2 Generation of the anti-NIP ScFv antiserum: The protocol used to raise an antiserum to the affinity-purified anti-NIP ScFv is described in section 2.9.2. In Figs. 4.20-4.22, the anti-(anti-NIP ScFv) activity of the various bleeds are

compared. The tertiary bleed showed a similar titration curve to the secondary bleed. As a result, the rabbit was killed at this stage and its antiserum collected.

4.2.3.3 Serological identification of the pp6 gene product using the anti-NIP ScFv antiserum: pp6 was transfected into the BHK cell line as previously described (section 4.2.1). 48hrs later, the cell population was analysed for expression of the gene product using the anti-NIP ScFv antiserum, essentially as described in section 2.8.2.2. The antiserum, at a dilution of 1:1000, was used as the primary probe, rather than the anti-c-myc. Also, a FITC-anti-rabbit IgG secondary antibody was used, at the manufacturer's recommended dilution. A typically stained cell is shown in Fig. 4.23. This experiment provided final, conclusive proof that, the desired intrabody was, indeed, being expressed, apparently in the cytosol. On the other hand, it was equally clear that, the poor expression levels indicated by the anti-c-myc probe were genuine and not due to selective degradation of the c-myc tag. Using the anti-NIP ScFv antiserum under these conditions, only 5 times as many cells were detected as positive for intrabody expression compared with when the anti-c-myc probe was used (see appendix B).

The anti-NIP ScFv antiserum was also used to probe four of the panel of 19 stably transfected cell lines (N8, N9, N10 and N12) in the belief that this probe was more sensitive than the c-myc probe. When these four cell lines were analysed, immunocytochemically, no conclusive evidence of pp6 expression was found. N9 did appear to be very slightly positive for expression but the fluorescence was too faint to document photographically.

As indicated by Fig. 4.14, cell extracts had been prepared, according to section 2.8.3.1, from some of the stable transfectants (N1-N12) during their initial propagation. These were probed with the antiserum by blotting as described in sections 2.8.3.3-2.8.3.5. All extracts were negative (results not shown). In retrospect, and aside from the low expression level of the pp6 intrabody, this is not too surprising. Greenman *et al.* (1996) have reported that NP40 lysates of cell lines stably transfected with an intrabody contained very little detectable ScFv. However, much more ScFv was detected when whole cell lysates were generated using SDS sample buffer.

Fig. 4.20

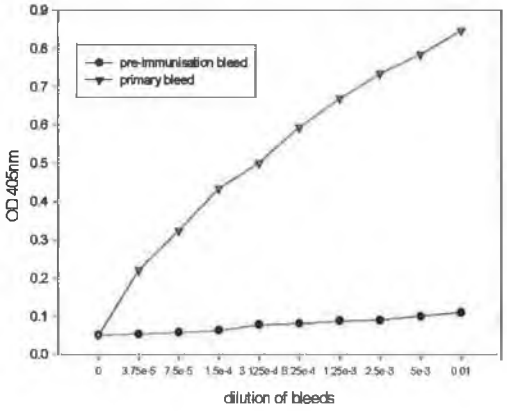


Fig. 4.21

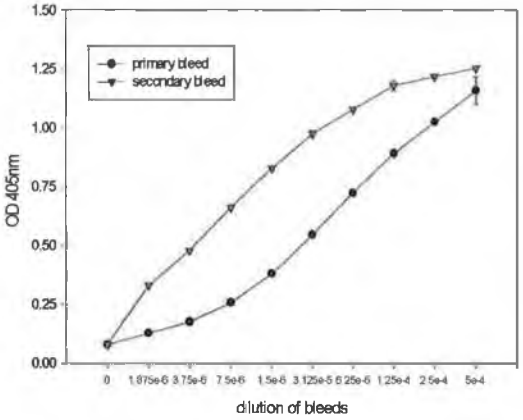
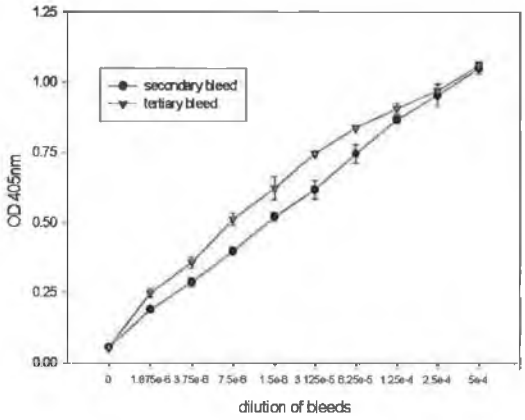


Fig. 4.22



Figs. 4.20-4.22: *A rabbit was immunised with the affinity-purified anti-NIP ScFv, as described in section 2.9.2. The anti-(anti-NIP ScFv) activity of the various bleeds was titrated by ELISA. The tertiary bleed showed a titre of at least 1:500,000 (Fig. 4.22) and was much higher in all probability. (The titre is defined here as the highest dilution used which gave an OD 405nm reading significantly greater than the background). Also, the tertiary and secondary bleeds gave similar titration curves. Therefore, the rabbit was killed at this stage and its antiserum harvested (section 2.9.2).*

ELISA: The ELISA was carried out as described in section 2.8.1.1. A 10μg/ml solution of the affinity-purified anti-NIP ScFv was used as the coating antigen. An alkaline phosphatase conjugated to goat anti-rabbit IgG was used as the secondary antibody at the manufacturer's recommended dilution.

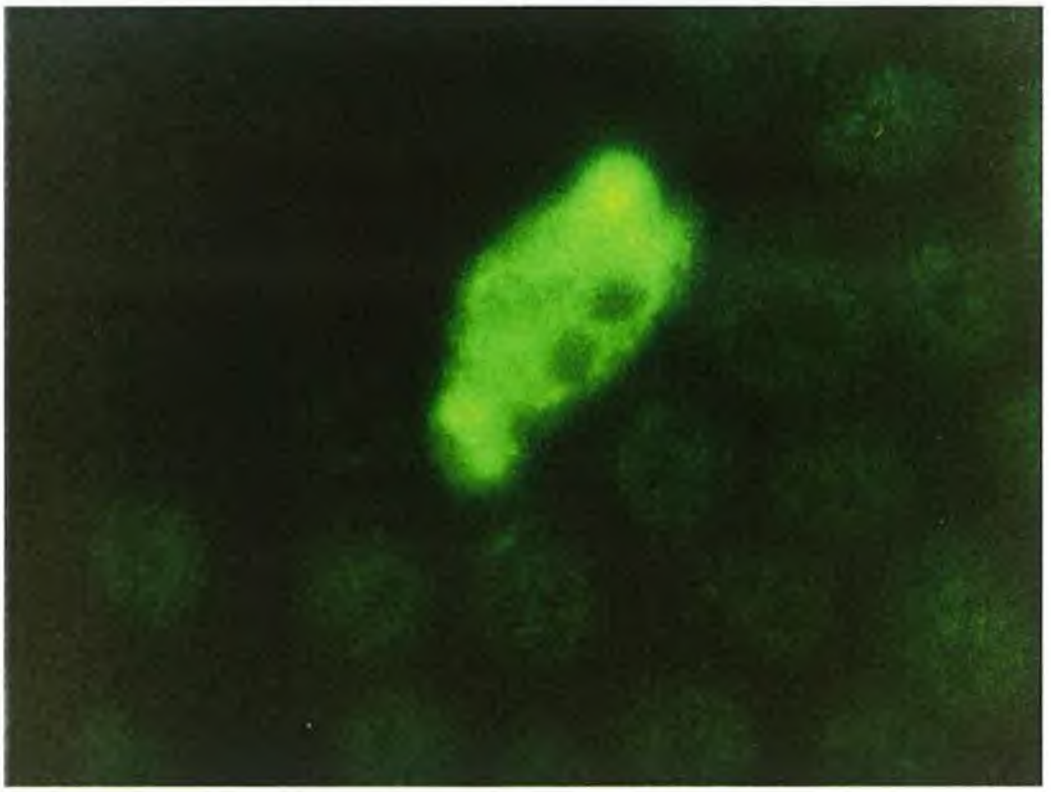


Fig. 4.23: *A typical BHK cell expressing pp6 gene product is presented in the above photograph (1000x magnification). The gene product was detected with the anti-NIP ScFv antiserum at a 1:1000 dilution and a FITC-anti-Rabbit IgG at the manufacturer's recommended dilution. At any higher concentration, the anti-NIP ScFv antiserum gave rise to unacceptable levels of non-specific background. Again, the gene product was in evidence throughout the entire cell, as was expected for a small cytosolic protein.*

4.3 Conclusions

The ultimate aim of all the intrabody studies reported in this thesis (this chapter and chapter 5) was to generate an intrabody library, for the purposes described in section 1.4.4.1. To this end, the studies recorded in this chapter were carried out to validate a generic strategy for targeting Nissim ScFvs to eukaryotic cytosols. The results of this study, while not as good as one might have hoped, give cause for guarded optimism with regard to the feasibility of intrabody libraries. In so far as one can tell using light microscopy, an anti-NIP intrabody was clearly expressed in the cytoplasm. However, the expression levels appear to be very low presumably due to a very short intracellular half-life. While, it would be advantageous to be able to argue that, the low expression levels observed are atypical and due to the particular intrabody under study, this is not the case. The anti-NIP intrabody appears to be quite average, neither over- nor under-expressed relative to the majority of potential intrabodies. This statement will be justified in chapter five.

The low expression levels observed have obvious ramifications for any application of cytosolic intrabody libraries - in order to favour phenotypic knockout of any intracellular protein, one ideally requires that the intrabody be expressed in as high an intracellular concentration as possible. This ensures that the antibody-antigen equilibrium favours formation of the antigen:antibody complex. High expression levels are, probably, a prerequisite for any application of intrabodies, but the requirement is more severe in the case of libraries. This is because, while intrabodies to a specific protein can be chosen to have a very high affinity, intrabody libraries will only contain intrabodies of mediocre affinity at best. Hence, one would have to conclude, based on current ScFv designs, that cytosolic intrabody libraries are not feasible.

This being the case, why the optimism? One remains optimistic for a number of reasons. Firstly, these studies have not addressed the feasibility of targeting intrabody libraries to other sub-cellular locations. As discussed in section 1.4.1, the cytosol appears to be the most inimical environment for intrabody expression. Based on observations reported in the literature, targeting of the nucleus, ER or mitochondria - locations of equal importance to

the cytosol- might be expected to lead to far superior expression levels (Biocca and Cattaneo, 1995).

Secondly, there is reason for cautious optimism that, in the long term, cytosolic intrabody libraries may be feasible. Current problems with the expression of ScFvs in the cytosol appear to stem from the fact, that natural variable regions have evolved over millions of years for expression in the endoplasmic reticulum, an organelle quite different in character from the cytoplasm. It may be possible, however, starting from 'natural' ScFvs (i.e. those based on naturally occurring variable regions) to engineer artificial 'variable region molecules' capable of high level expression in the eukaryotic cytosol.

Before discussing potential strategies to accomplish this, the assumption is made that the major reason for the short half-life of cytosolic intrabodies is the inability of the ScFv to fold in this environment. More specifically, the ScFv cannot form the requisite disulfide bonds for variable domain stability. This is a reasonable working assumption (Worn and Pluckthun, 1998), backed up by some experimental evidence (Biocca *et al.*, 1995). Such abnormal ScFvs are likely to be quickly degraded in the cytosol by the ubiquitin-dependent proteolytic pathway (Dice, 1987). By analogy with observations made in the bacterial cytoplasm, these abnormal ScFvs may also aggregate to form insoluble inclusion bodies.

Another potential reason for the low expression levels is that, the cytoplasm and the associated lysosomes are the major intracellular site of protein degradation. Even correctly folded ScFv may be expected to degrade with far greater rapidity in this environment than the endoplasmic reticulum.

These considerations suggest that, it is necessary to make the ScFv folding reaction more thermodynamically and kinetically favourable. It would also make sense to increase the solubility of the native ScFv and its folding intermediates. Studies to increase the resistance of the intrabody to proteolysis would be desirable, also. Such ideas are guiding the molecular evolution of ScFvs, using a combination of rational design and random mutagenesis.

The intrabody design presented in this chapter has both good and bad aspects with regard to its susceptibility to proteolysis. The intrabody is expected to have a Met amino acid at its n-terminus. This is expected to

protect the protein from cytosolic proteolysis, to some extent (Dice, 1987). However, as noted earlier in the chapter, the c-myc tag at the intrabodies C-terminus can be expected to decrease the half-life of the intrabody. The C-terminal extensions possessed by a protein are known to be an important determinant of half-life (Bowie and Sauer, 1989) and it is worth noting that, one generally applicable strategy for increasing the stability of cytosolic intrabodies has been the use of selected C-terminal tags. The addition of a constant domain to the ScFv has been advocated (Mhashilkar *et al.*, 1995; Jannot and Hynes, 1997). Schouten *et al.*, (1996 and 1997) reported that the addition of the ER-retention tetrapeptide KDEL increased the expression of two different ScFvs in the cytosol of tobacco protoplasts. The reason(s) for the increases in intrabody stability were not determined. One suggestion (Schouten *et al.*, 1997) was that, these C-terminal tags actually prevent the action of exonucleases acting at this terminus.

Pluckthuns group has provided the most elegant examples of ScFv evolution. Two relevant examples are discussed below.

Nieba *et al.* (1997) have pointed out that, the variable/constant domain interfaces of the whole antibody are exposed in the ScFv format. This interface is very hydrophobic and these authors have demonstrated most elegantly that, replacing selected hydrophobic residues with hydrophilic ones increases, dramatically, the solubility of the folding intermediates. The result was that, the particular ScFv under study, in mutated form, accumulated in the cytoplasm of *E. coli* at 25 times the level of the wild-type.

More recently, two papers (Proba *et al.*, 1998; Worn and Pluckthun, 1998) have demonstrated that, when the disulfide-forming cysteine residues of two different ScFvs were replaced with valine - alanine, the mutant ScFv showed a greater thermodynamic stability than the reduced wild-type. These authors have suggested that, this may be a generically applicable strategy to increase intrabody stability in the cytoplasm of eukaryotic cells.

Two major points are evident from these studies. Firstly, the expression of individual ScFvs in harsh ectopic environments, such as bacterial and eukaryotic cytosols, *may* be improved by molecular evolution techniques. Secondly, in the long term, these techniques may lead to the design/selection of 'super-frameworks'. These frameworks would offer

increased resistance to proteolysis and improved folding characteristics (Hudson, 1998; Worn and Pluckthun, 1998). They would form the basis of 'CDR-graft' libraries (Worn and Pluckthun, 1998), suitable for use as intrabody libraries.

The tools developed and reported in this chapter (the anti-c-myc and the anti-NIP ScFv antiserum, the cytochemical assays for intrabody expression level), in conjunction with the library technology to be reported in the next chapter, may make the anti-NIP intrabody an attractive model with which to test out some of the ideas promulgated above. Derivatives of the anti-NIP intrabody could be created and their expression level relative to the parental anti-NIP intrabody assayed by transfection into the BHK cell line followed by immunocytochemical analysis with the anti-(anti-NIP ScFv) probe. Specifically, in the immediate future it will prove possible to remove the c-myc tag from the anti-NIP intrabody described in this chapter and, subsequently, determine whether expression levels are improved. Taking this approach to its logical conclusion one could equip the anti-NIP moiety with a variety of C-terminal tags and assay their expression levels. In this regard, a review of the literature to determine stabilising C-terminal tags may facilitate the rational redesign of the anti-NIP intrabody. Alternatively a random approach could be pursued, whereby the MIK2 primer is redesigned to contain random sequences of nucleotides at its 5' end. The product of a PCR with such a mixture of primers would be a gene library encoding the anti-NIP ScFv moiety attached to random c-terminus tags. Stabilising tags would then be isolated from the library. A similar approach may reveal stabilising n-terminus tags.

Equally, one could redesign the anti-NIP intrabody so that it contains the amino acid substitutions suggested by the work of Nieba (1997) and Proba *et al.* (1998). Indeed, given that the amino acid substitutions suggested by these authors were selected in bacteria, it may prove fruitful to repeat their experiments and select stabilising substitutions in the eukaryotic cytosol. Many other approaches to improve the expression level of the anti-NIP intrabody can be articulated, including rational design based on the structures and sequences of intrabodies reported as successfully knocking out their complementary ligands.

The value of such studies should not be underestimated. Ideally, the design modifications resulting from studies with the anti-NIP intrabody could be generally applied to increase the stability of any cytosolic intrabody. If the resulting increases in intrabody stability were sufficiently large, cytosolic intrabody libraries based on such designs may become feasible. Given the poor expression levels observed in this study, this may be a lot to expect. However, even modest increases in cytosolic stability might make all the difference to the effectiveness of intrabodies designed from antibodies selected to specific proteins, e.g., the HIV protein Rev with consequences for, in this instance, therapeutic efficiency. The worst case scenario would be where the design modifications proved to be effective only for the anti-NIP intrabody. Even if this came to pass, the types of techniques used to improve the stability of the anti-NIP intrabody could be applied to any other relevant intrabody in order to provide 'tailor-made' modifications resulting in higher expression levels.

It may be argued that the potential for remodelling cytosolic intrabodies (libraries or particular intrabodies) in order to enhance their stability has been overstated. Whether or not this is so will only become apparent by putting the above ideas into practice. In the case of intrabodies targeted to specific proteins, it may also be argued that attempts to enhance their effectiveness by increasing their stability is futile to begin with, as it may be accomplished at the expense of binding site activity. The problem is acknowledged. Some of the proposed modifications such as C-terminal tags can reasonably be expected to increase stability without significantly reducing the binding activity of the intrabody. Problems may be expected to arise when one modifies framework residues. As noted in section 1.1.1.3, residues in the framework can affect the conformation of the CDRs. Equally, in some antibodies, framework residues may actually participate in binding antigen (section 1.1.4). Thus, mutating a framework or, perhaps, replacing a framework may or may not affect the binding activity of an intrabody. Thus, it is true to say, that not all mutations increasing the stability of a particular intrabody can be expected to result in enhanced phenotypic knockout. Notwithstanding, the selection of mutants, which enhance the stability of particular intrabodies is still considered relevant. While not all mutants

enhancing intrabody stability will increase the potential for phenotypic knockout, all mutations enhancing phenotypic knockout can be expected to result from an increase in stability. Thus, selecting mutants to increase stability may be a useful first step in screening for mutants enhancing phenotypic knockout.

Increasing the stability of cytosolic intrabodies is almost certainly a long-term aspiration. The strategies proposed to achieve this are not trivial; neither are they guaranteed success. However, there are many potential benefits to be gained, which may justify undertaking the relevant studies. The tools reported in this chapter may usefully play a role in these studies.

CHAPTER 5

GENERATION OF AN INTRABODY LIBRARY

It was highlighted in the previous chapter that, high expression levels of the constituent intrabodies are an essential pre-condition, if the intrabody library concept is ever to become feasible. An equally important pre-requisite is that the library be sufficiently diverse and the work to be presented in this chapter stemmed from a consideration of how diverse it should be.

Diversity is hugely important for the simple reason that, the more diverse the library, the greater the expected affinities of its constituents for any randomly chosen antigen. For any intrabody, it is the combination of its affinity and intracellular concentration that will determine whether it successfully 'knocks-out' its complementary ligand. Looked at from this perspective, it may seem that the library could never be too diverse. While this may very well be true, in practice there is an upper limit to the number of different specificities, which can be transfected into a population of eukaryotic cells (one can only work with a limited number of cells). This upper limit is $1x10^7$ specificities, approximately, so as an initial goal, the potential for generating a cytosolic intrabody library, containing $1x10^7$ different specificities, was investigated.

Viewed retrospectively, in light of the results presented in chapter four, this work might seem premature. However, while there is no hope of applying the cytosolic library to be described in this chapter, as originally envisaged, it may yet prove useful for molecular evolution studies. Furthermore, the technologies and ideas developed during these studies should aid the design of intrabody libraries targeted to locations other than the cytosol.

5.1 Exonuclease-based PCR-cloning: An initial consideration of the problem of constructing a cytosolic intrabody library suggested that, two modifications to the strategy used to generate the anti-NIP intrabody were required. The first modification was trivial. Plasmid prepared from the entire Nissim library rather than one isolate was required for the PCR (see Fig. 4.2).

The second modification involved finding a more efficient alternative to TA-cloning of PCR product. The reason for this was as follows. If the intrabody library was to contain $1x10^7$ specificities, a library consisting of at least $1x10^7$ independent transformants, containing insert in the correct orientation, was required. Referring to section 4.1.2, TA-cloning resulted in

only 350 transformants from 36ng of ligation reaction product. Furthermore, as only two of seven colonies tested contained the insert in the correct orientation, only 100 of the 350 colonies were relevant. This experiment used chemically competent cells. Even using the most efficient electroporation competent cells, one could only expect a 50-fold increase in the number of transformants obtained (5000 transformants per 36ng of ligation product). Thus, to create a library of the desired size using TA-cloning, the 'brute-force' cloning of $\approx$ 72µg of ligation product would be required. This was not practical and necessitated a 'PCR-cloning' strategy of greater efficiency than 'TA-cloning'.

The inefficiency of TA-cloning has as its root cause, a dependency upon very short (one base) complementary 'sticky-ends'. A review of the literature revealed that by careful choice of the sequences at the 5' end of PCR primers, much longer 'sticky-ends' could be generated by post-reaction modification of the PCR product. These strategies include 'ligase-free' subcloning (Schuldiner *et al.*, 1991), exonuclease-based strategies (Stoker, 1990; Kuijper *et al.*, 1992; Hsiao, 1993), restriction cut-back (Kaufman and Evans, 1990) and heterostagger cloning (Liu, 1996).

The strategy of Kuijper *et al.* (1992) was of particular interest as it was highly efficient, involved directed-cloning and produced very low non-recombinant backgrounds. This strategy as applied to create an intrabody library is depicted in Fig. 5.1. Two new primers designated MIKL1 and MIKL2 were designed. These differed from MIK1 and MIK2 in that they contained additional 5' sequences. Thus, they were expected to amplify the same sequence as the parental primers. It was important that these additional sequences were composed, primarily, of only three of the four possible nucleotides. The missing nucleotide was dTTP. As Fig. 5.1 makes clear, when the purified PCR product is incubated with T4 DNA polymerase, with dATP as the sole nucleotide present in the buffer, the 3'-5' exonucleolytic activity of the enzyme generates 13bp 'sticky-ends' on the PCR product. This is referred to as 'cut-back' of the product. The large size of these sticky-ends, combined with the fact that, their sequences differ on the sense and antisense strands, underlies the highly efficient, directed-cloning of the product.

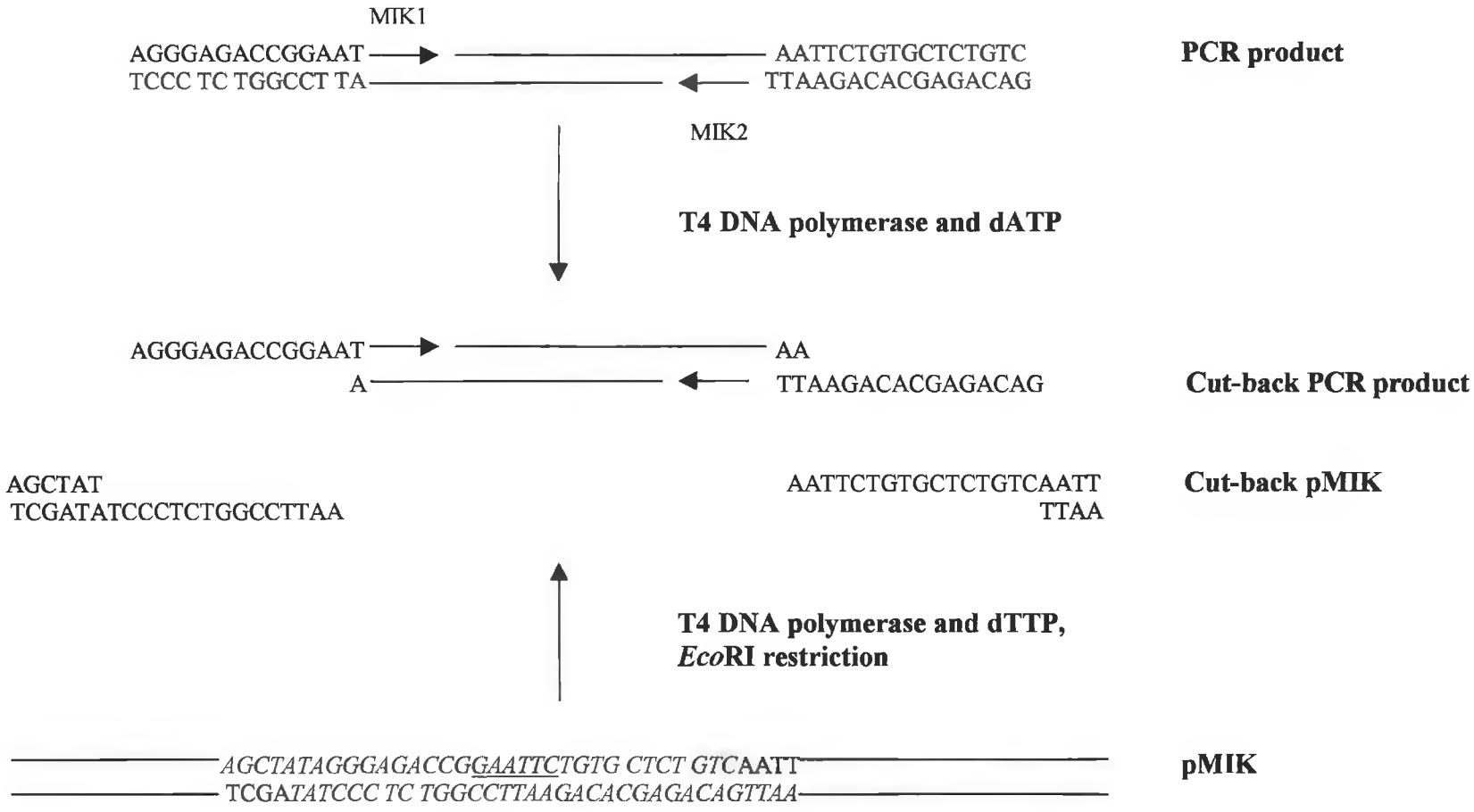


Fig. 5.1: *The exonucleolytic cloning strategy used to generate an intrabody library is depicted in the above diagram. pMIK is described in appendix A and section 5.1.1. The synthetic duplex DNA used to create pMIK is shown in italics with the EcoRI restriction site underlined. Sequences removed by the 3'-5' exonucleolytic activity of T4 DNA polymerase are shown in blue.*

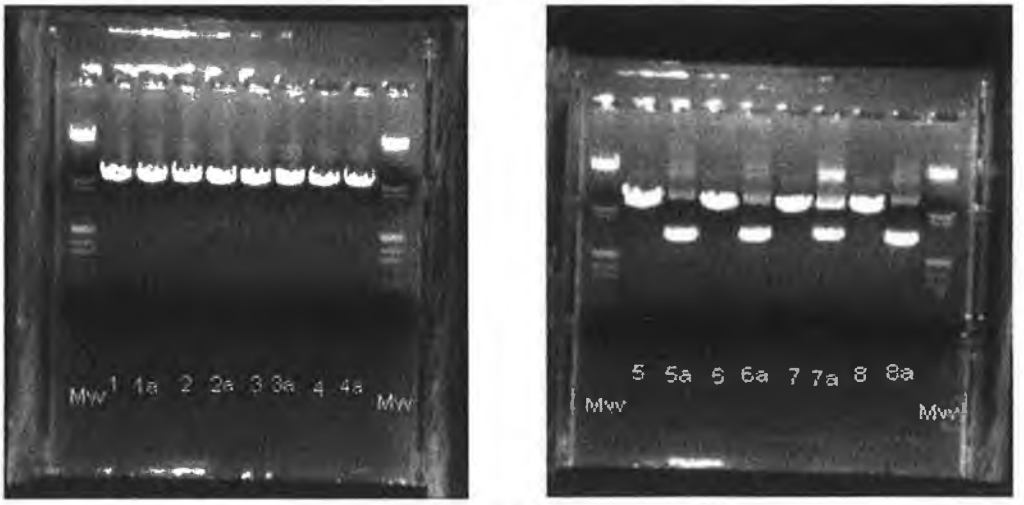
No mammalian expression vector was available that could be modified to produce complementary sticky-ends. Therefore, the eukaryotic expression vector pcDNA3.1+ (see appendix D for plasmid map and sequence) was modified by inserting duplex DNA into its multiple-cloning-site, and the resulting vector was termed pMIK (see appendix A). The sequence of the duplex DNA was chosen so that, restriction by *Eco*RI followed by cut-back, would reveal sticky ends complementary to those on the cut-back PCR product (Fig. 5.1).

5.1.1 Creation of pMIK: pcDNA3.1+ was transformed into *E. coli* Top10F' according to the manufacturer's instructions; one transformant was picked as a source of this plasmid. Ultrapure plasmid was purified from this clone according to section 2.7.2.2. pMIK was generated, by inserting a synthetic duplex DNA oligonucleotide into doubly-digested (*Hin*dIII and *Eco*RI) pcDNA3.1+, as described in section 2.7.5.1, and depicted in appendix A.

The synthetic duplex DNA inserted, was designed to contain two sticky-ends, one complementary to *Hin*dIII and one complementary to *Eco*RI sticky-ends. Although complementary, they did not have the ability to recreate these restriction sites. The synthetic DNA did, however, contain an internal *Eco*RI site. Thus, the recombinant plasmid was expected to have lost the unique *Hin*dIII site and retain a unique *Eco*RI site.

As noted in section 2.7.5.1, transformants had to be analysed by restriction digest in order to identify a transformant containing a recombinant plasmid. Screening for recombinants was carried out, by digesting plasmids in two separate reactions with *Hin*dIII and *Eco*RI. 8 transformants were screened in this fashion and the results are depicted in Fig. 5.2. In total, half of the colonies contained recombinant plasmids; one colony was maintained and its plasmid referred to as pMIK.

5.1.2 PCR: Initially, in order to test the two new primers, MIKL1 and MIKL2 were substituted for MIK1 and MIK2, respectively, in the PCR described in section 4.1.1. Fig. 5.3 demonstrates that these primers worked just as efficiently as their parental primers.



Figs. 5.2: *Plasmid preparations from 8 different transformants (numbered 1-8) were restricted with EcoRI or HindIII (lanes labelled a) in two separate reactions. It was evident that the plasmids 1-4 were non-recombinant as they were restrictable by both restriction enzymes. However, plasmids 5-8 were only restricted by EcoRI and were considered recombinant. That colony from which plasmid 5 was isolated was maintained and the plasmid was named pMIK. (The M_w markers are as described in Fig. 4.3).*

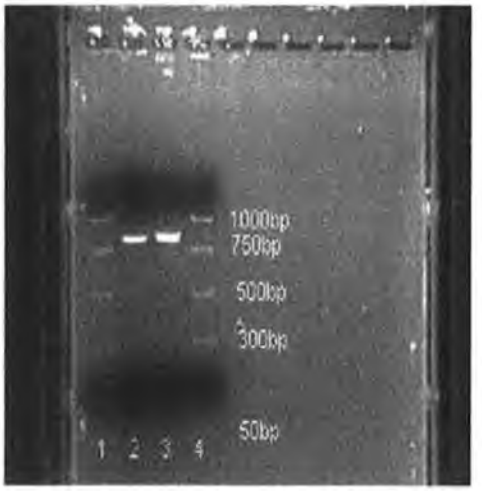


Fig. 5.3: *Two PCRs were set up, essentially as described in section 2.7.3.1. Both PCRs contained ultrapure pHen-Vλ + anti-NIP as template DNA. One PCR (lane 2) contained MIK1 and MIK2 as primers, while the other (lane 3) contained MIKL1 and MIKL2 as primers. The products of the PCRs were size-fractionated by gel electrophoresis. Both sets of primers gave a very similar product as expected. However, the product produced by amplification with MIKL1 and MIKL2 was slightly larger. This was as expected as the MIKL1 and MIKL2 primers are each ≈ 14bp longer than MIK1 and MIK2.*

In order to generate a 'PCR-library' of intrabody genes, suitable for exonucleolytic cloning, DNA was purified from the Nissim library, rather than one isolate. 50µl of Nissim library glycerol stock (10^8 bacteria) were grown overnight in 10ml of 2xTY medium supplemented with glucose and carbenicillin to final concentrations of 1% (w/v) and 100µg/ml, respectively. Plasmid was purified from this culture according to section 2.7.2.1. The plasmid was restricted, subsequently, with *Eco*RI, and gel purified according to section 2.7.2.1.

15 PCRs were set up, according to section 2.7.3.2, using this source of template DNA and MIKL1 and MIKL2 as primers. The reason for using *Pwo* polymerase was that, the 5' overhangs generated by *Taq* polymerase could have inhibited cut-back of the PCR product. A random sample (6) of the PCRs were analysed by gel electrophoresis (Fig. 5.4). They worked perfectly and all PCRs were combined. The reaction product was purified using a PCR cleanup kit, according to the manufacturer's instructions, and eluted in 60µl of TE buffer, pH 8.0. The concentration of purified PCR product was estimated by gel electrophoresis at 0.125mg/ml.

5.1.3 pMIK cut-back: pMIK was restricted with *Eco*RI and cut-back as described in sections 2.7.5.2 and 2.7.5.3, respectively. No analytical technique was available to assess the success or otherwise of this reaction. It was simply assumed to have occurred.

5.1.4 PCR cut-back: This was carried out according to section 2.7.5.4. Again the reaction was assumed to have occurred.

5.1.5 Ligation of cut-back pMIK with cut-back PCR: This reaction was carried out as described in section 2.7.5.5. This reaction was analysed by gel electrophoresis (Fig. 5.5) and appeared to be quite successful.

5.1.6 Transformation: Transformation of the product of the ligation reaction into *E. coli* XL1-Blue MRF' took place according to section 2.7.5.6. The size of the library was estimated, by plating, as $1.3x10^7$ independent clones.

Fig. 5.4

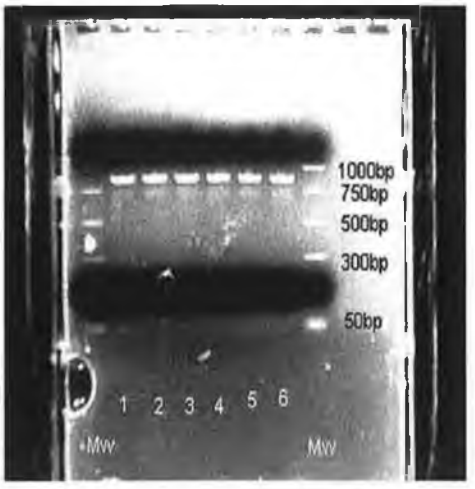
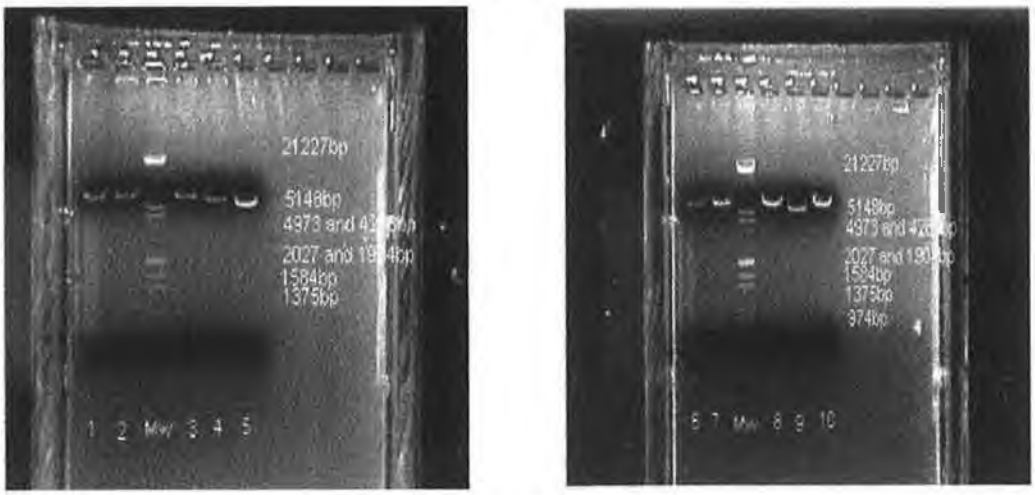


Fig. 5.5

Fig. 5.4: *15 PCRs were set up as described in section 2.7.3.2. Ultrapure plasmid DNA isolated from a sample of the Nissim library was used as the template DNA. MIKL1 and MIKL2 were used as forward and reverse primers, respectively. 6 of the PCRs were chosen at random, and their product was size-fractionated by gel electrophoresis (lanes 1-6). The PCRs worked very well. Only one major band was evident and it occurred where one would expect an 810bp product to occur.*

Fig. 5.5: *Cut-back PCR product was ligated to cut-back pMIK as described in section 2.7.5.5. The product was restricted with EcoRV and, subsequently, size-fractionated by gel electrophoresis (lane 3). Cut-back pMIK was also run through the gel (lanes 1 and 2) as were molecular weight markers. This vector was expected to be ≈ 5432bp in size (this is the stated size of pcDNA3.1+), an expectation confirmed by the gel. The ligation product, as expected, appeared to be of higher molecular weight than the cut-back vector. The ligation reaction appeared to have gone to completion as indicated by the fact that only one major band was evident.*



Figs. 5.6: *Plasmids were purified from 10 different transformants and restricted with EcoRV (lanes numbered 1-10). Mw markers were also run on the gel, as indicated in the margins of the pictures. The plasmids were expected to be ≈ 6,250bp in size if they were recombinant. If non-recombinant, they were expected to be ≈ 5432bp in size. All the above plasmids appear to be recombinant by this criterion. Note that, in one case (plasmid run in lane 9) the plasmid appeared to contain an EcoRV site in its insert, as two bands are evident in this lane.*

In order to determine the percentage of these colonies that contained recombinant plasmids, plasmids from 10 colonies were purified, restricted with *Eco*RV (unique site in both pcDNA3.1+ and pMIK), and their size was estimated by gel electrophoresis (Fig. 5.6). All 10 colonies appeared recombinant according to this criterion. Subsequently, a mock ligation was set up (no cut-back PCR product) exactly as per section 2.7.5.5. The product of this ligation reaction was transformed into *E. coli* XL1-Blue MRF' exactly as described in section 2.7.5.6. The number of clones which resulted was only 0.2% of the number of clones in the library. This suggested that the library consisted of 99.8% recombinant transformants.

Overall, it was evident that the strategy used to generate the library had been extremely successful from the points of view of library size and percentage recombinants. This success can be attributed to three factors. Firstly the use of the highly efficient exonucleolytic cloning strategy, secondly, the use of a large scale ligation reaction, and thirdly, the use of high efficiency (>$5x10^9$ transformants per μg of DNA) electrocompetent cells. The problem of describing the diversity of this library is discussed in the next section.

5.2 Library diversity: Having produced the library, an attempt was made to estimate how diverse it was. The diversity of any library is bound at its upper end by the diversity of its starting material. The starting material for the intrabody library was the Nissim library which, probably, contains $1x10^8$ specificities. For the sake of argument, this discussion proceeds under the assumption that, it was composed of this number of different specificities.

Having established an upper limit, it should be noted that, at each step of library construction you lose diversity. In this section, in as far as is possible, this loss of diversity is modelled.

5.2.1 Bacterial growth: 50μl of the Nissim library glycerol stock ($1x10^8$ bacteria) was used as the starting material for the intrabody library. Probablity theory indicates that this sample of the Nissim library did not contain $1x10^8$ different specificities. The number of specificities can be calculated as a function of sample size, for any degree of certainty (Fig. 5.7). The relevant model is:

$$1\text{-}P = (z/1x10^8)^n$$

where P is the probability of picking, in n attempts, a new specificity from $1x10^8$ specificities, and z is the number of different specificities already picked.

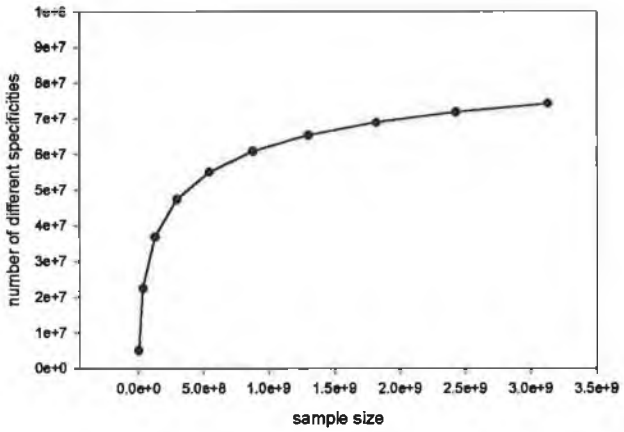


Fig. 5.7: *This graph depicts, at the 95% confidence level, the number of specificities (z), as a function of sample size (s) when one is sampling the Nissim library. Values of z (z_1, z_2, z_3....) were calculated for each value of n (n_1, n_2, n_3....). The corresponding values of s (s_1, s_2, s_3....) were calculated using the following formula:* $s_r = z_1 + \Sigma((n_{r-1}+n_r)/2)z_r$.

Using this graph, it is evident that, the sample of the Nissim library chosen, contained $3x10^7$ different specificities. Bacterial growth occurred in the presence of glucose to inhibit expression of ScFv. As a result, it is assumed that no selective pressure reduced library diversity during the growth of the sample. The final plasmid preparation was expected, therefore, to contain $3x10^7$ different specificities at an average frequency of $1/3x10^7$.

5.2.2 PCR: The next step involved selecting a sample of plasmids for use as templates during the PCR. Fig. 5.8 similar to Fig. 5.7 depicts diversity as a function of sample size. It was calculated exactly as for Fig. 5.7, from the formula:

$$1\text{-}P = (z/3x10^7)^n.$$

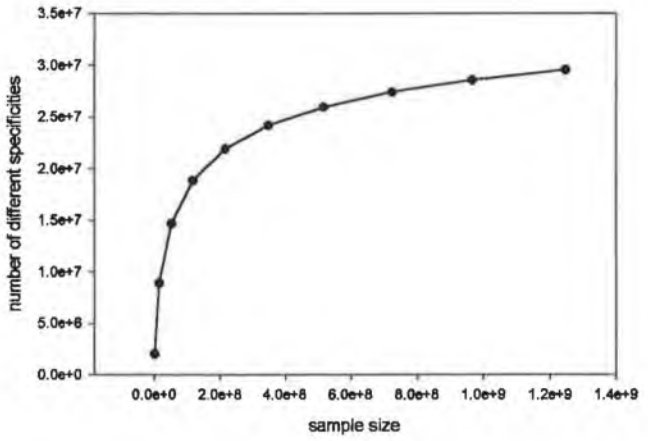


Fig. 5.8: *This graph depicts the number of specificities (z) as a function of sample size (s), when one is sampling from a population of plasmids encoding $3x10^7$ different specificities. See the legend to Fig. 5.7 for further details.*

The sample size for the PCR reaction was $1.5x10^7$ template molecules (15 PCR reactions each containing $1x10^6$ template molecules). The above graph indicated that this sample only contained $9x10^6$ different specificities.

Unfortunately, no data is available with regards to the efficiency of the PCR reactions. Therefore any loss of diversity during amplification cannot be modelled. This discussion proceeds on the assumption that, the PCR reactions were 100% efficient, and that no loss of diversity occurred. Although this may seem to be a major assumption, this is balanced, to some extent, by the use of a very cautious 95% confidence limit while modelling the loss of diversity due to sampling effects.

5.2.3 Ligation: It is assumed that no significant loss of diversity occurred during the ligation step. This is a reasonable assumption, given the number of molecules ligated in comparison with the number of specificities, and the efficiency of ligation.

5.2.4 Transformation: The loss of diversity upon transformation is again due to sampling effects. The relevant equation is:

$$1-P = (z/9x10^6)^n$$

and the relevant graph of number of specificities (z) versus the sample size (s) is depicted in Fig. 5.9.

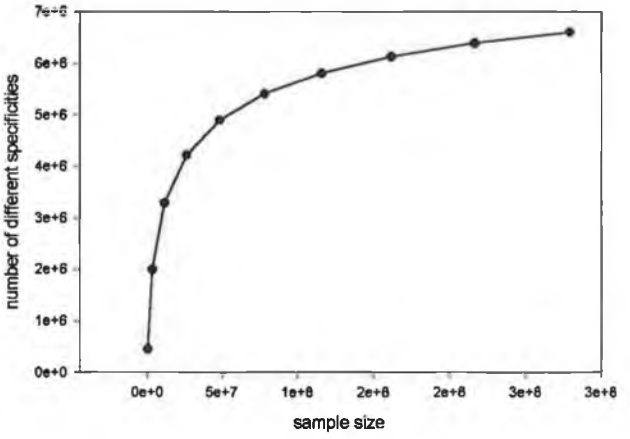


Fig. 5.9: *The graph of number of specificities (z) versus sample size (s), calculated from 1-P = $(z/9x10^6)^n$ is depicted. See the legend to Fig. 5.7 for further details.*

This graph allowed one to estimate the final diversity of the intrabody library. The number of transformants (equivalent to sample size) was $1.3x10^7$. Thus, the library was expected to contain $3x10^6$ different specificities.

5.3 Immunocytochemical analyses

The gene products expressed by the library were identified serologically, by immunocytochemistry, for two reasons. Firstly, to ensure that the gene products were expressed as and where expected. Secondly, it was considered that such studies would allow one to infer whether the expression levels attained by the anti-NIP intrabody were average or otherwise. If this intrabody was atypically stable or unstable, one would expect a decrease or an increase, respectively, in the percentage of BHK cells expressing detectable levels of library gene products.

These studies were carried out as described in section 4.2. A maxiprep of library DNA was prepared as described in section 2.7.2.2. The DNA was transfected into the BHK cell line as described in section 2.5.8.1, using

previously optimised conditions (section 4.2.1). After 48 hrs the resulting cell population was probed with either anti-c-myc or the anti-NIP antiserum, as previously described in section 4.2.2 and 4.3.2.3, respectively.

Figs. 5.10 and 5.11 show typical cells expressing library gene product as detected with anti-c-myc (Fig. 5.10) and anti-NIP antiserum (Fig. 5.11). These studies led to three conclusions. Firstly, they indicated that the gene products were, apparently, ScFvs expressed in the cytosol. They allowed the positive identification of the gene products as cytosolic intrabodies. Secondly, it was evident that, the anti-NIP antiserum appeared to have a general ability to detect ScFvs from the Nissim library. This was quite a surprising observation at first. However, the Nissim library contains only the one light chain variable domain. It is this feature, shared by all Nissim ScFvs, which, presumably, facilitates the detection of Nissim ScFvs by the antiserum. Thirdly, and without carrying out a detailed experimental comparison, the number of BHK cells expressing library gene product was strongly reminiscent of the numbers expressing pp6 gene product. This was taken as evidence in favour of the anti-NIP intrabody being relatively average, in terms of expression level.

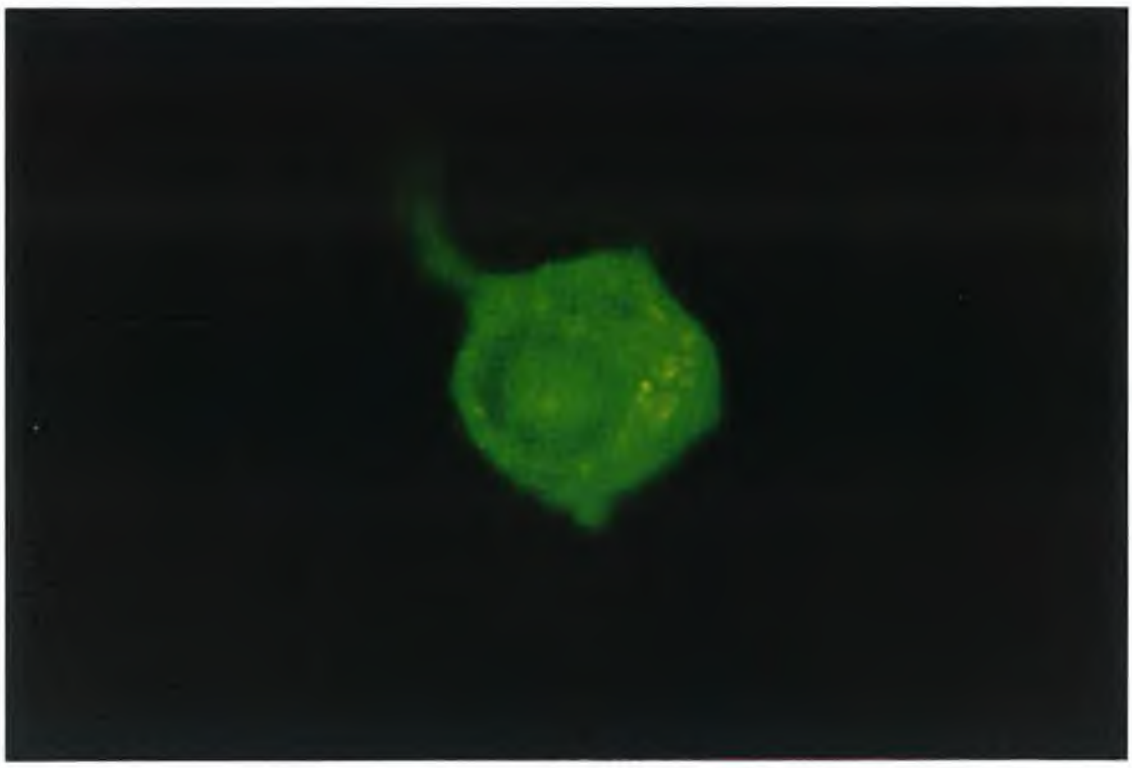


Fig. 5.10: *This photograph was obtained by probing a BHK cell population, which had been transfected with intrabody library DNA, with anti-c-myc as previously described (see section 4.22 and Fig. 4.10). The entire cell was diffusely stained, the nucleus to a lesser extent than the cytoplasm.*

Fig. 5.11: *This photograph was obtained under similar conditions to that presented in Fig, 5.10. However, the anti-NIP antiserum was used as a probe (see section 4.3.2.3 and Fig. 4.23) rather than the anti-c-myc. The staining pattern here is quite similar to that of Fig. 5.10. In contrast to that photograph, two distinct areas of the nucleus are stained to a lesser extent than the remaining portion. These two areas were not identified but may represent the nucleoli.*

5.4 Conclusions

The first conclusion reached from the series of experiments reported in this chapter, is more pertinent to chapter four and the topic of intrabody expression levels, than it is to this chapter and the topic of diversity. This was that the expression levels achieved by the anti-NIP intrabody were representative of those achievable by Nissim ScFvs, in general. Quite clearly, based upon expression levels alone, it is not feasible to use this cytosolic library as envisaged initially.

Equally, a number of useful points emerged, with respect to the creation of highly diverse intrabody libraries. Firstly, the library construction strategy presented in this chapter is, probably, sufficiently efficient to allow the construction of highly diverse intrabody libraries. While, the model presented to evaluate loss of diversity during library construction suggests a severe decrease in diversity (from $1x10^8 \rightarrow 3x10^6$ specificities), the final diversity is not too far removed from the initial target of $1x10^7$ specificities. This suggests that the target diversity can be achieved relatively easily using the proposed strategy. One could, for example, combine the above library with other libraries generated from differing sources of variable regions such as splenic mRNA or the Griffiths library (Griffiths *et al.*, 1994). Indeed, the model suggests that these would be a far superior source of variable regions than the Nissim library, due to their greater inherent diversity ($1x10^{10}$). In fact, using a primary source of such diversity, it should be possible to generate an intrabody library containing $1x10^7$ specificities in one go.

Having made this point, it must be said that the target diversity proposed for intrabody libraries at the start of this chapter was underestimated considerably. This became evident while modelling the loss of diversity due to sampling effects during library construction. If, as originally proposed, one should aim to transfect $1x10^7$ specificities into a population of eukaryotic cells, sampling effects will require that the intrabody library contains not $1x10^7$ specificities, but $4x10^7$ specificities.

A final point, which emerged during the modelling of diversity, was that, the generation of diversity during the PCR process would greatly aid in the creation of highly diverse intrabody libraries. The Nissim ScFv library

was constructed in such a fashion. The heavy chain CDR3 was added, randomly, during amplification of the 50 germline V_H segments (Nissim *et al.*, 1994).

Based on the experimental evidence presented, and assumptions made, in this chapter and chapter four, the following conclusion can be drawn. The generation of intrabody libraries of $> 4x10^7$ specificities is very possible. In order to accomplish this diversity, one of two different approaches would ideally be used. Firstly, one could sub-clone from a phage-display library containing a greater diversity of variable regions than the Nissim library. The Griffith library may be suitable. Preferably, one would create a semi-synthetic intrabody library using, e.g., the technology used to create the Nissim library rather than sub-cloning from this library. While, intrabody expression in the cytosol is not presently feasible, these techniques, in the short-term, should allow the creation of intrabody libraries targeted to a more suitable sub-cellular location, such as the endoplasmic reticulum. This may allow a clearer evaluation of the potential of the strategy.

Finally, it may be speculated that, the cytosolic library, and the technology used to generate it, may prove useful for the molecular evolution of stable cytosolic intrabodies, a topic previously discussed in section 4.3. The actual technology for generating a library is essential to some of the strategies mentioned in section 4.3 aimed at redesigning the anti-NIP intrabody so that it accumulates in the cytoplasm at high levels. The library technology outlined in this chapter is eminently suited to this purpose. A final idea, which might be worth pursuing, is as follows. It was proposed in section 4.3, that certain frameworks may be inherently more stable than others in the cytosol. Such 'super-frameworks' might form the basis for a second-generation intrabody library. It was suggested that one strategy for generating such frameworks was to mutate the anti-NIP intrabody and assay for enhanced accumulation in the cytosol. Perhaps certain frameworks are more stable than others, and the possibility that the anti-NIP framework may not be an ideal template from which to mould 'super-frameworks' must be entertained. One way to choose an improved template might be to select from the intrabody library created during the course of these studies, those intrabodies that are most stably expressed. It is entirely feasible that this could be accomplished by

transfecting the library into BHK cells, probing the population of cells with anti-c-myc, and selecting those cells most intensely stained, with a FACS (fluorescence-activated cell sorter). Selected ScFvs might be further improved (in terms of expression levels achieved) by expressing their heavy chain variable domain with a library of light chain variable domains and repeating the procedure. The selected ScFv would then be used in place of the anti-NIP intrabody in the mutagenesis studies suggested in section 4.3.

CHAPTER 6

OVERALL CONCLUSIONS

At the start of this thesis two major aims were outlined. The first aim was to initiate a study on antibody polyreactivity with particular reference to antibodies raised by *in vitro* immunisation. The second aim was to investigate the feasibility of generating intrabody libraries as a source of therapeutic proteins or, perhaps, a tool for functional genomic analysis.

It is felt that both of these studies provided much useful information, which can be summarised as follows. The polyreactivity studies (chapter 3) provided clear proof, that IVI may generate polyreactive Mabs. On a more basic level, potential problems which may prevent the unbiased screening of antibody repertoires, that is to say screening without under- or over-estimating the extent of polyreactive antibodies, were highlighted. An under-estimation may be expected if the antibody repertoire was screened while part of a complex biological matrix. Unless one is careful, however, the process of purifying the antibody repertoire may create polyreactive antibodies from monoreactive ones, therefore over-estimating the extent of polyreactivity. This may lead, in turn, to a distorted perception of the role of polyreactive antibodies in immune and, perhaps, non-immune phenomena.

The intrabody studies (chapters 4 and 5) were equally informative. Three main conclusions were reached. Firstly, the results suggest that the possibility of creating and utilising cytosolic intrabody libraries, or, certainly, those based on the Nissim ScFv design, can be disregarded. Creating a sufficiently diverse library (chapter 5) is not a problem, but intrabody expression in the cytosol or, more precisely, the lack of it (chapter 4) is a serious problem. Secondly, it was also pointed out, that intrabody expression levels could be expected to be higher in many other sub-cellular locations, such as the endoplasmic reticulum. It was proposed that the intrabody library concept could possibly be more fully evaluated by generating an intrabody library targeted to this organelle. Thirdly, a plethora of strategies aimed at improving the expression levels of cytosolic intrabodies were articulated. The tools developed during the course of this work (anti-NIP intrabody, anti-NIP antiserum, the assay for intrabody expression levels and the library strategy developed) can now be used to investigate these strategies.

Finally, it is worth noting, that the concept of antibody polyreactivity impinges upon the practical use of antibodies. In particular, the feasibility of

the intrabody library concept is dependent not just upon the expression levels and diversity of its constituent intrabodies. It is also dependent upon their monospecificity. The techniques used in chapter 3 for investigating polyreactivity can now be applied to evaluate the quality of naïve ScFv libraries, such as the Nissim library, in terms of the extent to which they are composed of polyreactive antibodies. This in turn may affect the feasibility of intrabody libraries.

CHAPTER 7

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APPENDICES

pcDNA 3.1+

GCGTTTAAAC TTAAGCTTGG TACCGAGCTC GGATCCACTA GTCCAGTGTG GTGGAATTCT
CGCAAATTTG AATTCGAACC ATGGCTCGAG CGTAGGTGAT CAGGTCACAC CACCTTAAGA

Double-digestion with *Eco*RI and *Hind*III

GCGTTTAAAC TTA
CGCAAATTTG AATTCGA

AATTCT
GA

heteroduplex DNA

AGCTATA GGGAGACCGG AATTCTGTGC TCTGTC
TAT CCCTCTGGCC TTAAGACACC AGACAGTTAA

pMIK

Ligate the synthetic duplex DNA to the restricted pcDNA 3.1+ to generate pMIK

GCGTTTAAAC TTAAGCTATA GGGAGACCGG AATTCTGTGC TCTGTCAATT CT
CGCAAATTTG AATTCGATAT CCCTCTGGCC TTAAGACACC AGACAGTTAA GA

Appendix B: Percentages of transiently transfected cells expressing gene products.

Throughout chapter four, the percentage of transiently transfected cells (NIH3T3, CHO or BHK) expressing detectable levels of either β-galactosidase from the pCH110 reporter plasmid, or anti-NIP intrabody from the pp6 plasmid were reported. (The structures and sequences of all plasmids are depicted in appendix D). These percentages are collated in Table A2.

β-galactosidase, which is stably expressed in mammalian cells, is expressed from the sv40 early promoter. This promoter allows efficient transcription in all three cell lines used (Pharmacia Biotech Technical Services, personal communication). Thus, the percentage of cells expressing detectable levels of β-galactosidase (as determined by enzyme cytochemistry - section 2.8.2.1) was taken as the transfection efficiency for that cell line.

Cells expressing detectable levels of the anti-NIP intrabody were enumerated by immunocytochemistry (section 2.8.2.2) and this value used to calculate the percentage of cells expressing detectable levels of anti-NIP intrabody as described in the footnote to page 181. For any given cell line, the ratio of the percentage of cells expressing detectable levels of anti-NIP intrabody to the percentage of cells expressing detectable levels of β-galactosidase was taken as a measure of the efficiency of expression of intrabody.

	β-galactosidase	anti-NIP intrabody (anti-c-myc probe)	anti-NIP intrabody (anti-NIP ScFv antiserum)
NIH3T3	0.1%	0.02%	ND
CHO	1%	0%	ND
BHK	10-30%	0.05%	0.25%

Table A2: *The percentages of cells (NIH3T3, CHO or BHK) expressing detectable levels of β-galactosidase, from pCH110, were determined by enzyme cytochemistry (section 2.8.2.1). The percentages of cells (NIH3T3, CHO or BHK) expressing detectable levels of anti-NIP intrabody, from pp6, were determined by immunocytochemistry (section 2.8.2.2) using either an anti-c-myc probe (section 4.2.2) or an anti-NIP ScFv probe (section 4.2.3.3).*

The poor expression level of the anti-NIP intrabody in the CHO and BHK cell lines was attributed to rapid degradation of the protein. It could not be attributed to poor transcription as pp6 expresses the anti-NIP intrabody from the CMV immediate-early promoter, which is very active in the three cell lines used in this study (Invitrogen Technical Services, personal communication).

Appendix C: Biological Solutions.

2-Amino-2-Methyl-1-Propanol buffer: 740μM $MgCl_2$, 0.01% (v/v) Triton X-405, 0.1% (w/v) NaN_3, 9.85% (v/v) 2-amino-2-methyl-1-propanol.

Alkaline lysis solution 1: 25mM Tris.Cl (pH 8.0), 50mM glucose, 10mM EDTA.

Alkaline lysis solution 2: 0.2M NaOH, 1% (w/v) sodium dodecyl sulphate.

Alkaline lysis solution 3: 3M $KC_2H_3O_2$, 11.5% (v/v) glacial acetic acid.

Coating buffer: 10mM K_2HPO_4, 5mM KH_2PO_4, 1mM EDTA, 0.14M NaCl, 0.05% (w/v) NaN_3.

Coomassie Blue stain solution: 0.1% (w/v) Coomassie Blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid.

Cut-back buffer: 66mM Tris.Cl (pH 7.9), 134mM $KC_2H_3O_2$, 20mM $MgC_2H_3O_2$, 10mM dTT, 0.1% (w/v) gelatin, 400μM dTTP or dATP.

Destain solution: 10% (v/v) methanol, 10% (v/v) acetic acid.

Dissection Hank's Balanced Salt Solution: 1.5mM $CaCl_2$, 5.4mM KCl, 344μM K_2HPO_4, 483μM $MgCl_2$, 410μM $MgSO_4$, 0.137M NaCl, 5.5mM glucose, 0.01% (w/v) Phenol Red, 50ng/ml gentamicin, 2.5μg/ml amphotericin.

Electrophoresis buffer: 25mM Tris, 0.2M glycine, 0.1% (w/v) sodium dodecyl sulphate.

Fix solution: 0.1M phosphate buffer (pH 7.3), 0.2% (v/v) glutaraldehyde, 0.05M EGTA, 2mM $MgCl_2$.

Gey's A: 0.654M NH_4Cl, 0.025M KCl, 637μM K_2HPO_4, 27.7mM glucose, 0.02% (w/v) Phenol Red, 2.5% (w/v) gelatin.

Gey's B: 20.7mM $MgCl_2$, 6mM $MgSO_4$, 31mM $CaCl_2$.

Homogenising buffer: 60mM Tris.Cl (pH 6.8), 0.1M dTT, 800μM EDTA, 2% (w/v) sodium dodecyl sulphate, 1μg/ml aprotinin, 1μg/ml pepstatin, 50μg/ml TLCK.

Ligation buffer: 0.1M Tris.Cl (pH 7.8), 10mM $MgCl_2$, 20mM dTT, 20μM dATP.

Lysis buffer: 0.1M Tris.Cl (pH 8.0), 1mM KCl, 525μM $MgCl_2$, 0.5% (v/v) NP-40, 1μg/ml aprotinin, 1μg/ml pepstatin, 50μg/ml TLCK.

Rinse solution: 0.1M phosphate buffer (pH 7.3), 2mM MgCl2, 0.01% (w/v) sodium deoxycholate, 0.02% (v/v) NP-40.

Sample buffer: 0.06M Tris.Cl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue.

Solution A: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide.

Solution B: 1.5M Tris.Cl (pH 8.8), 0.4% (w/v) sodium dodecyl sulphate.

Solution C: 0.5M Tris.Cl (pH 6.8), 0.4% (w/v) sodium dodecyl sulphate.

Stain solution: See Rinse solution with 1mg/ml 5-bromo-4-chloro-3-indolyl phosphate, p-toluidene salt, 0.165% (w/v) sodium ferricyanide, 0.165% (w/v) sodium ferrocyanide.

Transfer buffer: 25mM Tris, 0.2M glycine, 20% (v/v) methanol.

Tris/EDTA buffer: 10mM Tris.Cl (pH 8.0), 1mM EDTA.

Wash buffer: 10mM Tris.Cl (pH 7.4), 0.14M NaCl, 0.005% (w/v) Thimerosal, 0.1% (v/v) Tween 20.

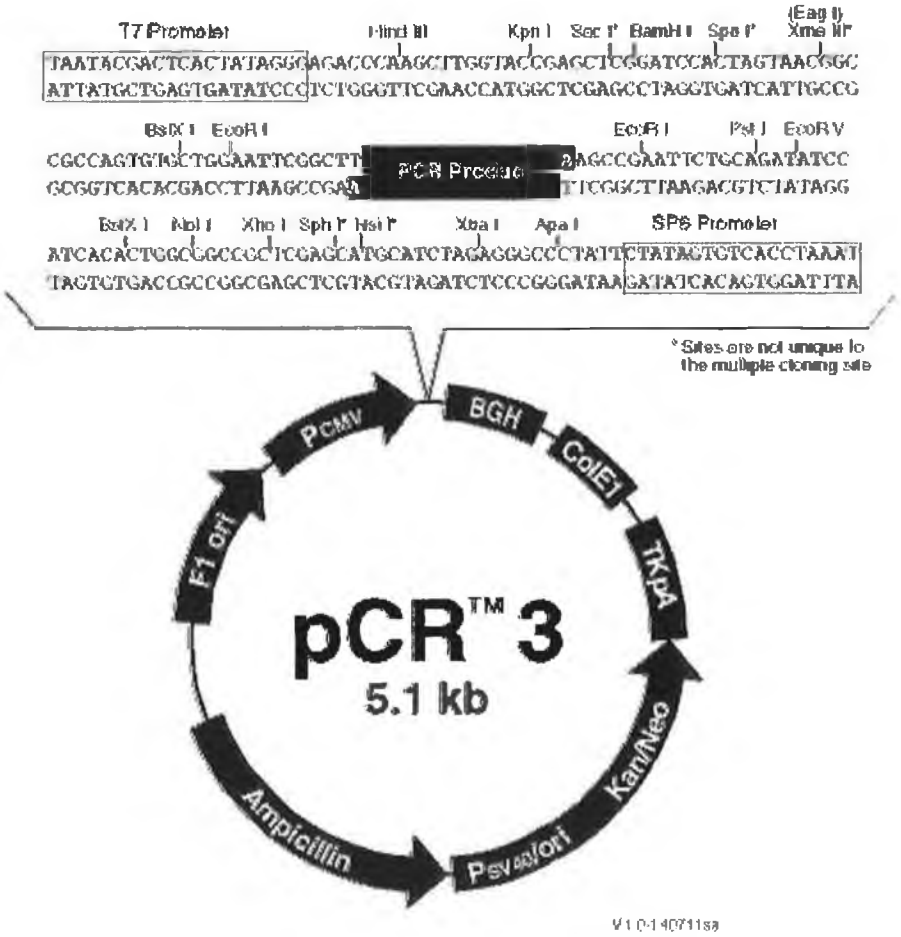
Appendix D: Commercial vectors used during the course of this study.

The following pages include plasmid maps and sequences for the three commercial vectors used during the course of this study. These are pCR3-Uni, pcDNA3.1+ and pCH110. The maps and sequences of pCR3-Uni and pcDNA3.1+ are available on the world-wide-web at www.invitrogen.com (both vectors are supplied by Invitrogen, DeSchlep, Netherlands). The sequence of pCH110 was obtained from the GenBank database, available on the world-wide-web at www2.ncbi.nlm.nih.gov. The corresponding map was obtained from Clontech Laboratories, Palo Alto, California, U.S.A., who provide this plasmid.

pCR3-Uni: This vector was generated by removing a total of 32bp between the *Hin*dIII and *Eco*RI sites of the polylinker of pCR3 (plasmid map and sequence are provided below:

Comments for pCR™3
5058 nucleotides

CMV promotor: bases 1-598
Putative Transcriptional Start: bases 620-625
T7 promotor: bases 638-657
Multiple Cloning Site: bases 664-789
SP6 promotor: bases 774-791
BGH poly A: bases 796-1024
ColE1 origin: bases 1155-1738
TK poly A signal: bases 1923-2194
Kanamycin/Neomycin resistance: bases 2196-3191
SV40 promotor/origin: bases 3192-3549
Ampicillin Resistance: bases 3568-4599
F1 origin: bases 4600-5056



U.S. Headquarters
Tel: 1-800-955-6288
Fax: 1-610-597-6201

European Headquarters
Tel: +31 (0) 5945-15175
Fax: +31 (0) 5945-15312

□

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```
        10         20         30         40         50         60
□
GCGCGCGTTG ACATTGATTA TTGACTAGTT ATTAATAGTA ATCAATTACG GGGTCATTAG

        70         80         90        100        110        120
TTCATAGCCC ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC CCGCCTGGCT

       130        140        150        160        170        180
GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC ATAGTAACGC

       190        200        210        220        230        240
CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGACTATTT ACGGTAAACT GCCCACTTGG

       250        260        270        280        290        300
CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT TGACGTCAAT GACGGTAAAT

       310        320        330        340        350        360
GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA CTTTCCTACT TGGCAGTACA

       370        380        390        400        410        420
TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC ATCAATGGGC

       430        440        450        460        470        480
GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC GTCAATGGGA

       490        500        510        520        530        540
GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG TCGTAACAAC TCCGCCCCAT

       550        560        570        580        590        600
TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA GCTCTCTGGC

       610        620        630        640        650        660
TAACTAGAGA ACCCACTGCT TACTGGCTTA TCGAAATTAA TACGACTCAC TATAGGGAGA

       670        680        690        700        710        720
CCCAAGCTTG GTACCGAGCT CGGATCCACT AGTAACGGCC GCCAGTGTGC TGGAATTCTG

       730        740        750        760        770        780
CAGATATCCA TCACACTGGC GGCCGCTCGA GCATGCATCT AGAGGGCCCT ATTCTATAGT

       790        800        810        820        830        840
GTCACCTAAA TGCTAGAGCT CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT

       850        860        870        880        890        900
CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC

       910        920        930        940        950        960
TTTCCTAATA AAATGAGGAA ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG

       970        980        990       1000       1010       1020
GGGGTGGGGT GGGGCAGGAC AGCAAGGGGG AGGATTGGGA AGACAATAGC AGGCATGCTG

      1030       1040       1050       1060       1070       1080
GGGATGCGGT GGGCTCTATG GCTTCTGAGG CGGAAAGAAC CAGTGGCGGT AATACGGTTA

      1090       1100       1110       1120       1130       1140
TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC
```

```
      1150        1160        1170        1180        1190        1200
AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC CCCTGACGAG

      1210        1220        1230        1240        1250        1260
CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC

      1270        1280        1290        1300        1310        1320
CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT GCCGCTTACC

      1330        1340        1350        1360        1370        1380
GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT

      1390        1400        1410        1420        1430        1440
AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC

      1450        1460        1470        1480        1490        1500
GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA

      1510        1520        1530        1540        1550        1560
CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA

      1570        1580        1590        1600        1610        1620
GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA

      1630        1640        1650        1660        1670        1680
TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA

      1690        1700        1710        1720        1730        1740
TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG

      1750        1760        1770        1780        1790        1800
CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG

      1810        1820        1830        1840        1850        1860
TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC

      1870        1880        1890        1900        1910        1920
TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAACCT

      1930        1940        1950        1960        1970        1980
GAGGCTATGG CAGGGCCTGC CGCCCCGACG TTGGCTGCGA GCCCTGGGCC TTCACCCGAA

      1990        2000        2010        2020        2030        2040
CTTGGGGGGT GGGGTGGGGA AAAGGAAGAA ACGCGGGCGT ATTGGCCCCA ATGGGGTCTC

      2050        2060        2070        2080        2090        2100
GGTGGGGTAT CGACAGAGTG CCAGCCCTGG GACCGAACCC CGCGTTTATG AACAAACGAC

      2110        2120        2130        2140        2150        2160
CCAACACCGT GCGTTTTATT CTGTCTTTTT ATTGCCGTCA TAGCGCGGGT TCCTTCCGGT

      2170        2180        2190        2200        2210        2220
ATTGTCTCCT TCCGTGTTTC AGTTAGCCTC CCCCTAGGGT GGGCGAAGAA CTCCAGCATG

      2230        2240        2250        2260        2270        2280
AGATCCCCGC GCTGGAGGAT CATCCAGCCG GCGTCCCGGA AAACGATTCC GAAGCCCAAC

      2290        2300        2310        2320        2330        2340
CTTTCATAGA AGGCGGCGGT GGAATCGAAA TCTCGTGATG GCAGGTTGGG CGTCGCTTGG
```

```
      2350        2360        2370        2380        2390        2400
TCGGTCATTT CGAACCCCAG AGTCCCGCTC AGAAGAACTC GTCAAGAAGG CGATAGAAGG

      2410        2420        2430        2440        2450        2460
CGATGCGCTG CGAATCGGGA GCGGCGATAC CGTAAAGCAC GAGGAAGCGG TCAGCCCATT

      2470        2480        2490        2500        2510        2520
CGCCGCCAAG CTCTTCAGCA ATATCACGGG TAGCCAACGC TATGTCCTGA TAGCGGTCCG

      2530        2540        2550        2560        2570        2580
CCACACCCAG CCGGCCACAG TCGATGAATC CAGAAAAGCG GCCATTTTCC ACCATGATAT

      2590        2600        2610        2620        2630        2640
TCGGCAAGCA GGCATCGCCA TGGGTCACGA CGAGATCCTC GCCGTCGGGC ATGCTCGCCT

      2650        2660        2670        2680        2690        2700
TGAGCCTGGC GAACAGTTCG GCTGGCGCGA GCCCCTGATG CTCTTGATCA TCCTGATCGA

      2710        2720        2730        2740        2750        2760
CAAGACCGGC TTCCATCCGA GTACGTGCTC GCTCGATGCG ATGTTTCGCT TGGTGGTCGA

      2770        2780        2790        2800        2810        2820
ATGGGCAGGT AGCCGGATCA AGCGTATGCA GCCGCCGCAT TGCATCAGCC ATGATGGATA

      2830        2840        2850        2860        2870        2880
CTTTCTCGGC AGGAGCAAGG TGAGATGACA GGAGATCCTG CCCCGGCACT TCGCCCAATA

      2890        2900        2910        2920        2930        2940
GCAGCCAGTC CCTTCCCGCT TCAGTGACAA CGTCGAGCAC AGCTGCGCAA GGAACGCCCG

      2950        2960        2970        2980        2990        3000
TCGTGGCCAG CCACGATAGC CGCGCTGCCT CGTCTTGCAG TTCATTCAGG GCACCGGACA

      3010        3020        3030        3040        3050        3060
GGTCGGTCTT GACAAAAAGA ACCGGGCGCC CCTGCGCTGA CAGCCGGAAC ACGGCGGCAT

      3070        3080        3090        3100        3110        3120
CAGAGCAGCC GATTGTCTGT TGTGCCCAGT CATAGCCGAA TAGCCTCTCC ACCCAAGCGG

      3130        3140        3150        3160        3170        3180
CCGGAGAACC TGCGTGCAAT CCATCTTGTT CAATCATGCG AAACGATCCT CATCCTGTCT

      3190        3200        3210        3220        3230        3240
CTTGATCGAT CTTTGCAAAA GCCTAGGCCT CCAAAAAAGC CTCCTCACTA CTTCTGGAAT

      3250        3260        3270        3280        3290        3300
AGCTCAGAGG CCGAGGCGGC CTCGGCCTCT GCATAAATAA AAAAAATTAG TCAGCCATGG

      3310        3320        3330        3340        3350        3360
GGCGGAGAAT GGGCGGAACT GGGCGGAGTT AGGGGCGGGA TGGGCGGAGT TAGGGGCGGG

      3370        3380        3390        3400        3410        3420
ACTATGGTTG CTGACTAATT GAGATGCATG CTTTGCATAC TTCTGCCTGC TGGGGAGCCT

      3430        3440        3450        3460        3470        3480
GGGGACTTTC CACACCTGGT TGCTGACTAA TTGAGATGCA TGCTTTGCAT ACTTCTGCCT

      3490        3500        3510        3520        3530        3540
GCTGGGGAGC CTGGGGACTT TCCACACCCT AACTGACACA CATTCCACAG CTGGTTCTTT

      3550        3560        3570        3580        3590        3600
```

CCGCCTCAGG ACTCTTCCTT TTTCAATAAA TCAATCTAAA GTATATATGA GTAAACTTGG

3610 3620 3630 3640 3650 3660
TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG TCTATTTCGT

3670 3680 3690 3700 3710 3720
TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA GGGCTTACCA

3730 3740 3750 3760 3770 3780
TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA

3790 3800 3810 3820 3830 3840
GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC TTTATCCGCC

3850 3860 3870 3880 3890 3900
TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC AGTTAATAGT

3910 3920 3930 3940 3950 3960
TTGCGCAACG TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG

3970 3980 3990 4000 4010 4020
GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC

4030 4040 4050 4060 4070 4080
AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT GGCCGCAGTG

4090 4100 4110 4120 4130 4140
TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC ATCCGTAAGA

4150 4160 4170 4180 4190 4200
TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA

4210 4220 4230 4240 4250 4260
CCGAGTTGCT CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA

4270 4280 4290 4300 4310 4320
AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG

4330 4340 4350 4360 4370 4380
TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC ATCTTTTACT

4390 4400 4410 4420 4430 4440
TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA AAAGGGAATA

4450 4460 4470 4480 4490 4500
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4510 4520 4530 4540 4550 4560
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4570 4580 4590 4600 4610 4620
ATAGGGGTTC CGCGCACATT TCCCCGAAAA GTGCCACCTG ACGCGCCCTG TAGCGGCGCA

4630 4640 4650 4660 4670 4680
TTAAGCGCGG CGGGTGTGGT GGTTACGCGC AGCGTGACCG CTACACTTGC CAGCGCCCTA

4690 4700 4710 4720 4730 4740
GCGCCCGCTC CTTTCGCTTT CTTCCCTTCC TTTCTCGCCA CGTTCGCCGG CTTTCCCCGT

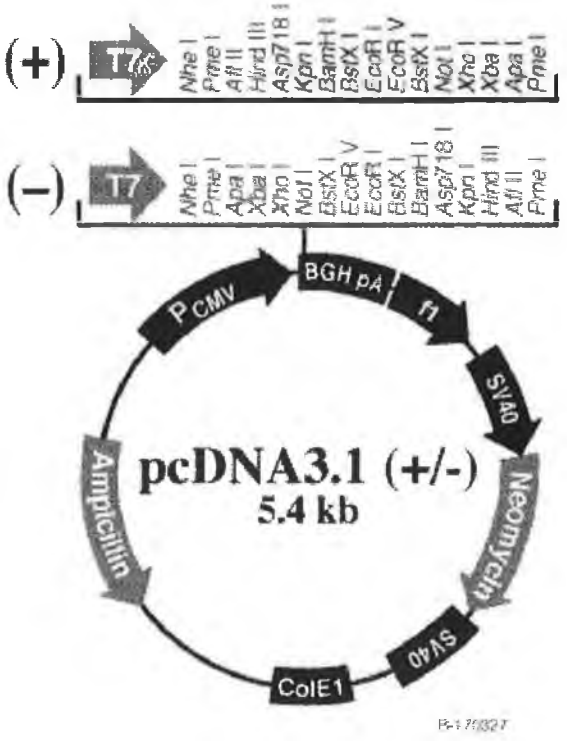
4750 4760 4770 4780 4790 4800
CAAGCTCTAA ATCGGGGGCT CCCTTTAGGG TTCCGATTTA GTGCTTTACG GCACCTCGAC

```
      4810        4820        4830        4840        4850        4860
CCCAAAAAAC TTGATTAGGG TGATGGTTCA CGTAGTGGGC CATCGCCCTG ATAGACGGTT

      4870        4880        4890        4900        4910        4920
TTTCGCCCTT TGACGTTGGA GTCCACGTTC TTTAATAGTG GACTCTTGTT CCAAACTGGA

      4930        4940        4950        4960        4970        4980
ACAACACTCA ACCCTATCTC GGTCTATTCT TTTGATTTAT AAGGGATTTT GCCGATTTCG

      4990        5000        5010        5020        5030        5040
GCCTATTGGT TAAAAAATGA GCTGATTTAA CAAAAATTTA ACGCGAATTT TAACAAAATA

      5050        5060        5070        5080        5090        5100
TTAACGCTTA CAATTTAC.. .......... .......... .......... ..........
```

pcDNA3.1+:

Comments for pcDNA3.1 (+)
5432 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1 reverse priming site: bases 1022-1039
BGH poly A: bases 1021-1235
f1 origin of replication: bases 1298-1711
SV40 promoter and origin: bases 1776-2101
Neomycin resistance gene: bases 2137-2931
SV40 polyadenylation signal: bases 2986-3358
ColE1 origin: bases 3618-4291
Ampicillin resistance gene: bases 4436-5297



The sequences of pcDNA3.1 (+/-) have been compiled from information in sequence databases, published sequences, and other sources. These vectors have not been completely sequenced. If you suspect an error in the sequences, please contact Invitrogen's Technical Services Department.

U.S. Headquarters
Tel: 1-800-955-6288
Fax: 1-760-603-7201

European Headquarters
Tel: +31 (0) 594 515 175
Fax: +31 (0) 594 515 312

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTT
AA
GCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAAC
AA
GGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGT
AC
GGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGT
TC
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CC
CCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
AT
GGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCC
TA
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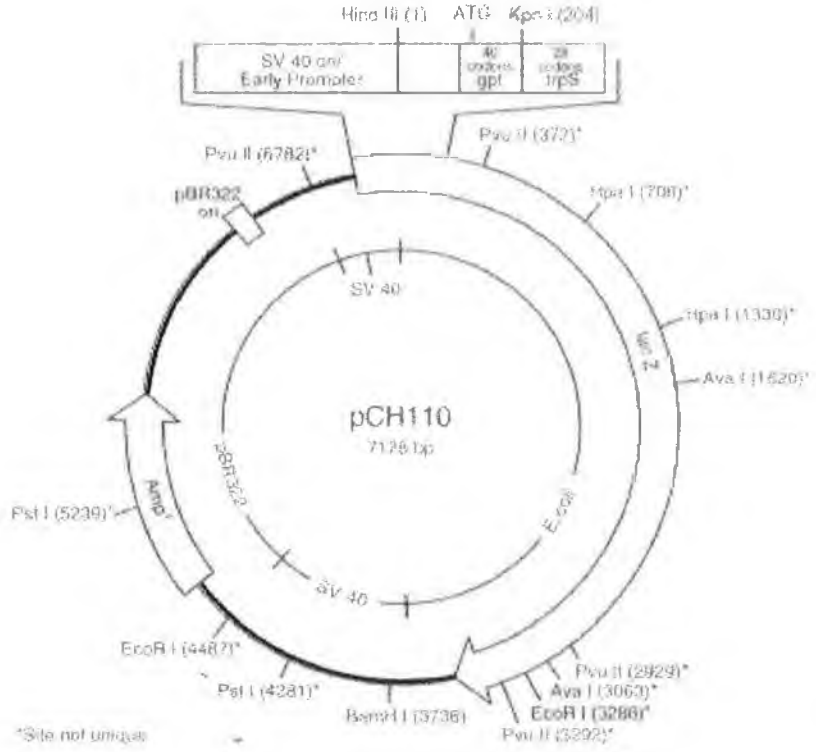
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